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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/45, 15/62, C07K 14/115, 14/12, 14/135, A61K 39/155, 39/165

A2

(11) International Publication Number: **WO 00/18929**

(43) International Publication Date: 6 April 2000 (06.04.00)

(21) International Application Number: PCT/EP99/07004

(22) International Filing Date: 20 September 1999 (20.09.99)

(30) Priority Data:

9820931.5	25 September 1998 (25.09.98)	GB
9906868.6	24 March 1999 (24.03.99)	GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: NOVEL COMPOUNDS

(57) Abstract

Heterochimeric proteins or immunogenic derivatives thereof are described comprising immunogenic fragments of RSV, PIV1, PIV2, PIV3, MV and MuV fusion and attachement glycoproteins. Such heterochimeric proteins may be expressed, in particular, in CHO cells and may be used in vaccine compositions to treat respiratory disorders such as those caused by paramyxoviridæ viral antigens.

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Novel Compounds

The present invention relates to recombinant heterochimeric paramyxoviridae glycoproteins and their expression in eukaryotic cells, particularly in Chinese Hamster Ovary (CHO) cells. The invention further relates to methods for constructing and expressing such heterochimeric proteins, intermediates for use therein, methods to optimize the codon usage of the nucleic acid sequences which encode such heterochimeric proteins and the use of the recombinant proteins as vaccines for the prevention of diseases caused by paramyxoviridae pathogens.

The mumps (MuV), Measles (MV), the parainfluenza type I (PIV1), type II (PIV2) and type III (PIV3) and the respiratory syncytial (RSV) virus belong to the paramyxoviridae family. The MuV is classified in the rubulavirus subclass, the MV is classified in the Morbillivirus subclass, the parainfluenza viruses (PIV1, PIV2 and PIV3) are classified in the paramyxovirus subclass while the RSV is attached to the pneumovirus subclass.

RSV is the most important cause of viral lower respiratory tract disease in infants and children. The fusion (F) and the attachment (G) protein which are both viral surface glycoproteins appear to be of potential value for the development of a vaccine against RSV.

The fusion protein F of RSV contains 574 amino acid residues; amino acids 1 to 21 correspond to the signal peptide and residues 525 to 549 to the membrane anchor domain. The molecule presents five potential sites for glycosylation. The F protein is synthesized as a 70 kDa precursor (F_0) which undergoes proteolytic maturation to yield the F_1 subunit (48 kDa) and F_2 (23 kDa) linked via disulfide bridges. The protein F, when injected into animals, leads to the production of neutralizing antibodies and may induce cytotoxic lymphocytes (CTLs).

The attachment or G protein of RSV contains 298 amino acid residues and is heavily glycosylated since half of its molecular mass (90 kDa) is contributed by

oligosaccharide side chains, chiefly in the form of O-linked sugars. It has been shown that the G protein, when injected into animals, provides protection against homologous but not heterologous subgroup virus challenge. This protein is extremely variable and there is only a stretch of 13 amino acid residues which is conserved in all RSV.

The PIV3 is second to RSV as a major agent of severe viral respiratory tract infections in infants. The fusion protein F of PIV3 contains 539 amino acid residues; amino acids 1 to 18 correspond to the signal peptide and residues 494 to 516 to the membrane anchor domain. The molecule presents 4 potential sites for glycosylation. The F protein is synthesized as a 70 kDa precursor (F_0) which undergoes proteolytic maturation to yield the F_1 (56 kDa) and F_2 (14 kDa) subunits linked via disulfide bridges. The protein F, when injected into animals, leads to the production of neutralizing antibodies. The F protein is involved in cell fusion during viral infection and carries an hemolysin activity. Used alone for immunization, the F protein generates an immune response which is insufficient to confer protection against a challenge with the virus. Complete protection is only acquired by concomitant immunization with the attachment protein HN, another glycoprotein of PIV3.

The protein HN carries hemagglutinin and neuraminidase activities. It is composed of 572 amino acids; its membrane anchor domain occurs in the N-terminal end of the molecule, between amino acid residues 32 and 53. Four potential sites for glycosylation have been identified. Injection of protein HN into animals generates an immune response and neutralizing antibodies. These antibodies however do not protect completely against a challenge with the virus. Full protection is obtained only by concomitant immunization with the F protein of PIV3.

The PIV1 virus was initially isolated from young children suffering from disorders of the lower respiratory tract. Infection with PIV1 causes the majority of cases of croup found for all infections caused by paramyxoviruses. Viral transmission of

PIV1 is by person to person contact or by aerosol, although the virus does not persist in the environment for long.

Like PIV2 and PIV3, the PIV1 virus has two surface glycoproteins, the fusion protein (F) and the attachment protein (HN). These two proteins are the priority targets for the development of a subunit vaccine, the properties of which would be to ensure protection of children from the very first months of life and to prevent reinfection, or at least to prevent the serious complications by restricting viral development to the upper respiratory tract where the consequences would be benign (common cold).

PIV2 also affects very young children and causes the same type of respiratory discorders, essentially croup, but of less severity. The PIV2 virus has two surface glycoproteins (F and HN), which are potential targets for the development of a subunit vaccine.

The measles virus is an extremely contagious agent which establishes itself in the epithelial cells of the respiratory tract, the oropharynx or the conjunctiva. The infection causes fever, cough, head-cold, conjunctivitis and a characteristic generalised rash.

There is no appropriate inactivated vaccine against measles but an effective attenuated live vaccine is available and is generally used in combination with the attenuated live vaccines against rubella and mumps. This live vaccine protects against the disease for at least 20 years. The measles virus has two surface glycoproteins, which are potential targets for the development of a subunit vaccine. The fusion protein (F) is a 550 amino acid long glycosylated molecule and, as for the other paramyxovirus, has to undergo proteolitic cleavage to yield F_1 and F_2 subunits that are linked via disulfide bridges. This molecule, which carries a haemolysin activity, generates an immune protective response when injected into animals. The attachment protein (H), is a 617 amino acid long glycosylated protein, which carries a hemagglutinin activity. This protein leads, when injected into

animals, to the production of neutralizing antibodies that are able to inhibit hemagglutination. This immune response protects the animal against a viral challenge.

The mumps virus is a pathogen causing the contagious infantile illness which consists of the inflammation of parotid glands. During the incubation period following infection, the virus replicates in the respiratory epithelium then disseminates into secretary ducts of the parotid glands. Other glands may become infected thereafter and numerous cases of meningitis have been reported. Among complications related to the infection, encephalitis is a serious one, with a mortality rate of about 1%; deafness cases have also been reported.

A vaccine against mumps is available: it is made of an attenuated live virus, produced by culturing infected embryonic chicken cells. The vaccine leads to the seroconversion in vaccinated individuals and protects against infection in more than 95% of seronegative persons. The vaccine thus reduces significantly the frequencies of complications.

In a number of cases, however, viral infection is not detected because the effects remain subclinical. Young children and aged people are most likely to develop complications from mumps infection. In view of the inherent risks related to the use of attenuated live vaccines, such as the potentiation of the illness upon natural surinfection in vaccinated individuals, it is desirable to improve the safety of the vaccine, particularly for the groups at risk.

The fusion protein F of mumps virus contains 538 amino acid residues; amino acids 1 to 26 correspond to the signal peptide and residues 483 to 512 to the membrane anchor domain. The molecule presents 7 potential sites for glycosylation. The F protein is synthesized as a 65-74 kDa precursor (F_0) which undergoes proteolytic maturation to yield the F_1 (58-61 kDa) and F_2 (10-16 kDa) subunits linked via disulfide bridges. The protein F is involved in cell fusion during viral infection, carries an haemolysin activity and plays a role for viral penetration into cells. It

does not however carry the antibody dependent cellular cytotoxicity (ADCC) as observed for another mumps virus glycoprotein, HN.

The protein HN (molecular weight 74-80 kDa) carries hemagglutinin and neuraminidase activities which are involved in virus attachment to cells and in the disruption of the host cell membranes. Protein HN (attachment protein or hemagglutinin-neuraminidase) generates neutralizing antibodies and appears important for the development of ADCC. Protein HN is composed of 582 amino acids; it carries a N-terminal anchor domain (residues 33 to 52) and 9 potential sites for glycosylation.

For the viruses considered above, it appears that concomitant immunization with both membrane glycoproteins F and HN, or G in the case of RSV, are required to achieve full protection in the animal model. Chimeric proteins containing both the F and G proteins of RSV, or the F and HN proteins of PIV3 have shown complete protection against RSV or PIV3 challenge in cotton rats (Brideau et al, J Gen Virol, 1989, 70 2637-2644 and Brideau et al, J Gen Virol, 1993, 74, 471-477).

WO9314207 (Connaught) describes heterochimeric proteins comprising RSV and PIV3 proteins including F(RSV)xHN(PIV3) and F(PIV3)xG(RSV) hybrids, and suggests that such proteins can be expressed from a variety of host cells including bacterial, mammalian, insect, yeast and fungal cells. The specific examples describe expression in insect Sf9 and High 5 cells and mammalian Vero cells. There is no specific disclosure of the use of CHO cells. The use of Sf9 and High 5 cells is also described by Du et al, BIO/TECHNOLOGY 12,1994, 813-818.

Homa et al (Upjohn), J Gen Virol, 1993, 74, 1995-1999 describes another heterochimeric protein, F(RSV)xHN(PIV3) expressed in insect cells using a recombinant baculovirus.

Homochimeric paramyxoviridae glycoproteins have also been described by several workers:-

WO8905823 (Upjohn) describes RSV FxG and GxF hybrids which can be expressed from bacterial, yeast, mammalian and insect cells. Example 7 describes the expression of an RSV FxG protein from CHO cells although there are no details of how successful such expression is.

WO8910405 (Upjohn) describes PIV3 FxHN and HNxF hybrids which can be expressed from bacterial, yeast, mammalian and insect cells. Example 6 describes the expression of a PIV3 FxHN protein from CHO cells, however no details are given quantifying the extent of expression and secretion.

Lehman et al (Upjohn), J Gen Virol, 1993, 74, 459-469 describes the expression of PIV3 FxHN in insect cells using recombinant baculovirus vectors as well as in CHO cells.

WO9306218 (SmithKline Beecham Biologicals) describes PIV3 FxHN hybrids which can be expressed in eukaryotic cells including vaccinia, CHO or Vero cells. Example B)2 describes the expression of a $Fs^{+}a^{-}xHNa^{-}$ hybrid in CHO cells and indicates that the product was almost evenly distributed between cells and medium. No details are however given quantifying the extent of expression and secretion.

WO9425600 (SmithKline Beecham Biologicals) describes MuV FxHN and HNxF hybrids which can be expressed in vaccinia, a mammalian cell (such as CHO) or a bacterial cell. Examples B) 3 and 4 describe the expression of $s^{+}FHNa^{-}xFa^{-}$ and $Fs^{+}a^{-}xHNa^{-}$ in CHO cells however no details are given describing the extent of expression and secretion.

Although this cited art may suggest that homochimeric paramyxoviridae glycoproteins can be expressed in a variety of cell lines including CHO cells it has now been discovered that in fact expression and secretion from CHO cells is not always successful and success cannot be predicted. Thus it has now been demonstrated that although a RSV F x G hybrid could be successfully expressed and

secreted in CHO cells, analogous homochimeric hybrids from PIV3 and MuV could not in fact be expressed in CHO cells in such manner that they could be purified from the supernatant in significant quantities.

Surprisingly, it has now been discovered that heterochimeric hybrids can be successfully expressed and secreted in both CHO and insect cells.

Accordingly in a first aspect the present invention provides a process for preparing a heterochimeric protein or an immunogenic derivative thereof comprising an immunogenic fragment of the fusion (F) protein of RSV, PIV1, PIV2, PIV3, MV or MuV and an immunogenic fragment of the attachment (G, HN or H) protein of RSV, PIV1, PIV2, PIV3, MuV or MV which process comprises expressing recombinant DNA encoding the heterochimeric protein or immunogenic derivative thereof in CHO cells and recovering the protein.

By heterochimeric protein is meant one that does not contain a fusion or attachment protein from the same pathogen.

This invention also provides novel heterochimeric proteins not previously described in WO 9314207 which can be prepared using the process of the present invention.

Thus, in a second aspect the present invention provides a heterochimeric protein or an immunogenic derivative thereof comprising an immunogenic fragment of the fusion (F) protein of RSV, PIV1, PIV2, PIV3, MV or MuV and an immunogenic fragment of the attachment (G, HN or H) protein of RSV, PIV1, PIV2, PIV3, MuV or MV, with the proviso that where one of the immunogenic fragments is derived from RSV F, RSV HN or PIV3 F, PIV3 HN, the other of the immunogenic fragments is derived from MuV F, MuV HN, MV F, MV H, PIV1 F,PIV1 HN, PIV2 F or PIV2 HN.

By an immunogenic fragment of the fusion (F) protein of RSV, PIV1, PIV2, PIV3, MV or MuV is meant a part of the protein which contains at least one antigenic

determinant capable of raising an immune response specific to the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV respectively. Included within this definition is the full length F protein, preferably however the immunogenic fragment is lacking the membrane anchor domain at its C-terminal end.

By an immunogenic fragment of the attachment protein (G, HN or H) of RSV, PIV1, PIV2, PIV3, MuV or MV is meant a part of the protein which contains at least one antigenic determinant capable of raising an immune response specific to the G protein of RSV, to the HN protein of PIV1, PIV2, PIV3, MuV or the H protein of MV respectively. Included within this definition is the full length G or HN protein, preferably however the immunogenic fragment is lacking the signal/anchor domain at its N-terminal end.

Preferably the heterochimeric protein is linked *via* an amino acid in the C-terminal part of the immunogenic fragment of the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV to an amino acid in the N-terminal part of the immunogenic fragment of the G protein of RSV, the HN protein of PIV1, PIV2, PIV3, MuV or the H protein of MV.

Suitably the heterochimeric protein commences at its N-terminal end with a signal sequence from the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV. Conveniently this will be part of the corresponding immunogenic fragment of the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV when this fragment is linked *via* its C-terminal end to the N-terminal end of the immunogenic fragment of the G protein of RSV, the HN protein of PIV1, PIV2, PIV3, MuV or the H protein of MV.

Alternative signal sequences may also be employed. For example, the heterochimeric protein suitably commences at its N-terminal end with a signal sequence of tissue plasminogen activator (TPA).

In order to enhance the level of expression the heterochimeric protein may further comprise a ubiquitin leader sequence which is suitably positioned after any signal sequence as hereinbefore described. Preferably the ubiquitin leader sequence is linked to the C-terminal end of the signal sequence of TPA.

Preferably the ubiquitin leader sequence is derived from yeast, for example as described in Ecker et al, J.Biological Chemistry, 1988, 264(13), 7715-7719.

Suitably a cleavage site is positioned between the C-terminal end of the ubiquitin sequence and the N-terminal end of the immunogenic fragment of the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV.

In order to facilitate chromatographic purification the heterochimeric protein suitably comprises a polyhistidine tail, for example as described in Hochuli et al, BIO/TECHNOLOGY, 1988, 1321-1325. The polyhistidine tail preferably comprises from 2 to 6 adjacent histidine residues which is suitably attached at the C-terminal end of the heterochimeric protein. Preferably a cleavage site is positioned between the polyhistidine tail and the C-terminal end of the immunogenic fragment of the G protein of RSV, the HN protein of PIV1, PIV2, PIV3, MuV or the H protein of MV.

The cleavage site for the ubiquitin sequence and/or the polyhistidine tail may be chemical or enzymatic and preferably is an enterokinase cleavage site, for example as described in LaVallie et al, BIO-TECHNOLOGY, 1993, 187-193.

Following expression and purification, treatment with an enterokinase will cleave off any ubiquitin and/or polyhistidine sequence releasing the desired heterochimeric protein.

Particular heterochimeric proteins of this invention include:
the F protein of RSV lacking its membrane domain linked at its C-terminal end to the HN protein of MuV lacking its signal/anchor domain herein referred to as:

$Fs^+a^-RSVxHNs^-a^-MuV$, as well as

Fs^+a^- PIV3 x HNs^-a^- MuV;

Fs^+a^- MuV x Gs^-a^-RSV; and

Fs^+a^- MuV x HNs^-a^-PIV3, and

immunogenic derivatives thereof.

The present invention also provides particular heterochimeric proteins which include:

$Fs^+a^-MuVxHs^-aMV$; or

$Fs^+a^-RSVxHNs^-a^-$PIV1; or

$Fs^+a^-RSVxHNs^-a^-$PIV2, and

imunogenic derivatives thereof.

The present invention also provides heterochimeric proteins comprising RSV and PIV3 proteins not specifically disclosed in WO9314207, which advantageously can be expressed from CHO cells.

These are:

Fs^+a^- (1-526) RSV x HNs^-a^- (70-572) PIV3;

Fs^+a^- (1-492) PIV3 x Gs^-a^- (69-298) RSV;

Fs^+a^- (1-526) RSV x HNs^-a^- (70-572) PIV3 bis;

Fs^+a^- (1-526) RSV x HNs^-a^- (70-572) PIV3 ent his, and

sTPA (1-21) UB (1-74) ent Fs^-a^- (24-526) x HN s^-a^-(70-572) PIV3, and

immunogenic derivatives thereof.

The heterochimeric proteins of the present invention are immunogenic. The term immunogenic derivative as used herein encompasses any molecule which is a heterochimeric polypeptide which is immunologically reactive with antibodies raised to the heterochimeric protein of the present invention or parts thereof or with antibodies recognising the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV, the G protein of RSV, the HN protein of PIV1, PIV2, PIV3, MuV, the H protein of MV, the RSV virus, the PIV1 virus, the PIV2 virus, the PIV3 virus, the MV virus or the MuV virus, or which, when administered to a human, elicits antibodies

recognising the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV, the G protein of RSV, the HN protein of PIV1, PIV2, PIV3, MuV, the H protein of MV, the RSV virus, the PIV1 virus, the PIV2 virus, the PIV3 virus, the MV virus or the MuV virus. In particular immunogenic derivatives which are slightly longer or shorter than the heterochimeric proteins of the present invention may be used. Such derivatives may, for example, be prepared by substitution, addition, or rearrangement of amino acids or by chemical modifications thereof including the coupling or for enabling the coupling of the heterochimeric proteins to other carrier proteins such as tetanus toxoid or Hepatitis B surface antigen. All such substitutions and modifications are generally well known to those skilled in the art of peptide chemistry.

Immunogenic fragments of the heterochimeric proteins which may be useful in the preparation of vaccines may be prepared by expression of the appropriate gene fragments or by peptide synthesis, for example using the Merrifield synthesis (The Peptides, Vol 2., Academic Press, New York, p3).

In a further aspect of the invention there is provided recombinant DNA encoding the heterochimeric protein of the invention. The recombinant DNA of the invention may form part of a vector, for example a plasmid, especially an expression plasmid from which the heterochimeric protein may be expressed. Such vectors also form part of the invention, as do host cells into which the vectors have been introduced.

In order to construct the DNA encoding a heterochimeric protein according to the invention, cDNA containing the coding sequences of the RSV, PIV1, PIV2, PIV3, MV or MuV fusion and attachment proteins and optionally of the ubiquitin, polyhistidine and enterokinase cleavage sites may be manipulated using standard techniques [see for example Maniatis T. et al Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y. (1982)] as further described hereinbelow.

In another aspect of the invention there is described a process of enhancing the protein expression in mammalian cells by optimization of the codon usage of the nucleic acids transfected therein. Optimization of the codon usage involves the replacement of at least one non-preferred or less preferred codon in a natural gene encoding a heterochimeric protein by a preferred codon encoding the same amino acid. Highly mammalian-expressed genes have C or G at their degenerative position (third base in the codon) whereas the RSV or PIV3-prevalent codons have A or T. At least one codon, and more prefereably all the codons of the RSV or PIV3 protein can be changed to fit at best the human usage, that is, the one (or ones) that is the most prevalent as shown below.

Ala: GCC	Cys: TGC	His: CAC	Met: ATG	Thr: ACC
Arg: CGC AGG CGG	Gln: CAG	Ile: ATC	Phe: TTC	Trp: TGG
Asn: AAC	Glu: GAG	Leu: CTG	Pro: CCC	Tyr: TAC
Asp: GAC	Gly: GGC	Lys: AAG	Ser: AGC TCC	Val: GTG

Each amino acid encoded by one of these codons are then considered humanised. The ratio between the number of humanised codons versus the total number of amino acids gives a percentage of humanisation as shown below.

1) $F_{RSV\ (1\text{-}526)original}$ 140/526 = 27%
2) $F_{RSV\ (1\text{-}423)humanised}$ + (424-526)original 403/526 = 77%
3) $F_{RSV\ (1\text{-}526)humanised}$ 489/526 = 93%
4) $F_{RSV\ (1\text{-}526)original}$ + $HN_{PIV3\ (70\text{-}572)\ original}$ 258/1029 = 25%
5) $F_{RSV\ (1\text{-}526)humanised}$ + $HN_{PIV3\ (70\text{-}572)\ original}$ 528/1029 = 51%
6) $F_{RSV\ (1\text{-}526)humanised}$ + $HN_{PIV3\ (70\text{-}572)\ humanised}$ 96%

The invention also provides DNA encoding a heterochimeric protein or immunogenic derivative thereof in which the codon usage of one or more nucleic acids has been substantially optimised and a process for expressing said DNA in a CHO or insect cell.

There have been a number of reports that have described a substantial amelioration of protein expression in mammalian cells after re-engineering the nucleic acid sequence of the heterologous protein to fit the codon usage found in highly expressed human genes (Haas J., Park E-C. and Seed B., Codon usage limitation in the expression of HiV-1 envelope glycoprotein, Current Biology, 1996, 6, n°3, 315-325 ; Kim C. H., Oh Y. and Lee T.H., Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells, Gene 199, 1997, 293-301 ; Zolotukhin S., Potter M. Hauswirth W.W. Guy J. and Muzyczka N. A Humanized green fluorescent protein cDNA adapted for high level expression in mammalian cells. J. of Virology, July 1996, 70, n°7, 4646-4654).

Vectors comprising such DNA, hosts transformed thereby and the truncated or hybrid proteins themselves, expressed as described hereinbelow all form part of the invention.

For expression of the proteins of the invention, plasmids may be constructed which are suitable either for transfer into vaccinia virus or transfection into CHO cells, insect cells or Vero cells. Suitable expression vectors are described hereinbelow. Preferably the proteins of the present invention are expressed in CHO or insect cells.

For expression in vaccinia a vaccinia transfer plasmid such as pULB 5213 which is a derivative of pSC11 (Chakrabati *et al*, Molecular and Cellular Biology *5*, 3403 - 3409, 1985) may be used. In one aspect the protein may be expressed under the control of the vaccinia $P_{7.5}$ promoter.

For expression in CHO-K1 cells a glutamine synthetase (GS) vector such as pEE14 may suitably be used so that the protein is expressed under the control of the major immediate early promoter of human cytomegalovirus (hCMV-MIE). Alternatively a vector which allows the expression of the coding module as a polycistronic transcript with the *neo* selection gene may suitably be used. In one preferred aspect the coding module is under the control of the Rous Sarcoma Long Terminal Repeat (LTR) promoter.

Preferably the plasmid for expression in CHO-K1 cells carries a GS expression cassette suitable for gene amplification using methionine sulphoximine (MSX). Alternatively the plasmid for expression in CHO-K1 cells carries a DHFR expression cassette suitable for gene amplification using methotrexate (MTX).

Preferably expression of the heterochimeric protein of the present invention is carried out in the presence of sodium butyrate and/or dimethyl sulphoxide (DMSO) which may enhance gene expression.

For expression in insect cells a shuttle vector such as pAcUW51 or pAcGP67 may be used. In one aspect the protein may be expressed under the control of the baculovirus p10 promoter or the polyhedrin promoter.

The expression system may also be a recombinant live microorganism, such as a virus or bacterium. The gene of interest can be inserted into the genome of a live recombinant virus or bacterium. Inoculation and *in vivo* infection with this live vector will lead to *in vivo* expression of the antigen and induction of immune responses. Viruses and bacteria used for this purpose are for instance: poxviruses (e.g; vaccinia, fowlpox, canarypox), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), herpesviruses (varicella zoster virus, etc), Listeria, Salmonella, Shigella, BCG. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

In yet another aspect of the invention there is provided a vaccine composition comprising a heterochimeric protein or immunogenic derivative thereof according to the invention in combination with a pharmaceutically acceptable carrier, a protein according to the invention for use in vaccinating a mammal and the use of a protein according to the invention in the preparation of a vaccine.

Optionally, and advantageously, the vaccine of the present invention is combined with other immunogens to afford a polyvalent vaccine. In a preferred embodiment the heterochimeric protein is combined with other subcomponents of RSV, PIV1, PIV2, PIV3, MuV or MV, e.g. the single proteins F, G, HN or H or homochimeric proteins such as RSV FxG, PIV3 FxHN or MuV FxHN.

In a particular aspect the invention further provides a vaccine composition comprising a protein according to the invention together with a suitable carrier or adjuvant.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller *et al*, University Park Press, Baltimore, Maryland, U.S.A., 1978. Encapsulation within liposomes is described, for example by Fullerton, U.S. Patent 4,235,877.

In the vaccine of the present invention , an aqueous solution of the protein(s) can be used directly. Alternatively, the protein, with or without prior lyophilisation, can be mixed, absorbed or adsorbed with any of the various known adjuvants. Such adjuvants include, but are not limited to, aluminium hydroxide, muramyl dipeptide and saponins such as Quil A. Particularly preferred adjuvants are MPL (monophosphoryl lipid A) and 3D-MPL (3 deacylated monophosphoryl lipid A) [US patent 4,912,094], optionally formulated with aluminium hudroxide (EP 0 689 454) or oil in water emulsions (WO 95/17210). A further preferred adjuvant is known as QS21 which can be obtained by the method disclosed in US patent 5,057,540. Use of 3D-MPL is described by Ribi *et al.* in Microbiology (1986) Levie *et al.* (eds)

Amer. Soc. Microbiol.Wash. D.C., 9-13. Use of Quil A is disclosed by Dalsgaard *et al.*,(1977), Acta Vet Scand, *18*, 349. Use of combined 3D-MPL and QS21 is described in WO 94/00153 (SmithKline Beecham Biologicals s.a). QS21 may be advantageously formulated with cholesterol containing liposomes, wherein 3D-MPL is present either in solution or incorporated in the membrane, as described in WO 96/33739.

As a further exemplary alternative, a heterochimeric protein of the invention or an immunogenic fragment thereof can be encapsulated within microparticles such as liposomes or associated with oil-in-water emulsions. Encapsulation within liposomes is described by Fullerton in US patent 4,235,877. In yet another exemplary alternative, a heterochimeric protein according to the invention or an immunogenic fragment thereof can be conjugated to an immunostimulating macromolecule, such as killed *Bordetella* or a tetanus toxoid. Conjugation of proteins to macromolecules is disclosed, for example by Likhite in patent 4,372,945 and Armor *et al.* in US patent 4,474,757.

The amount of the protein of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected that each dose will comprise 1-1000μg of protein, preferably 1-200 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects.

The following examples and the attached figures (explained below) illustrate the invention.

In the Figures:

Figure 34A shows the impact of humanisation on the level of expression of FrHNp, where:

FhHNE10 = product expressed by the pEE14FhHN transfected clone E10;

FhHNE7 = product expressed by the pEE14FhHN transfected clone E7;

FHNbis = product expressed by the pEE14FHN transfected clone;

+but = 2mM Nabutyrate has been added to the cell medium, 3 days before harvest;

pEE14 = negative control;

Fdroso = pruified Fa- (drosophila derived); the standard protein in this ELISA assay wherein 1ul of standard corresponds to 1ng of product.

Figure 34B shows humanisation impact on the level of expression of $F_{RSV}HN_{PiV3}$, where the level of expression was determined by ELISA. Fdroso = purified Fa- (drosophila derived) that is the standard protein in this ELISA assay, 1ul of standard corresponds to 1ng of product.

EXAMPLES

Example 1

In order to vaccinate with a single immunogen, heterochimeric DNA molecules were constructed combining extracellular domains of the F and the attachment protein for each virus. DNA constructs for the PIV3 and MuV have already been described in WO9306218 and WO9425600, respectively. The DNA molecule combining the extracellular domains of the RSV F and G proteins were constructed as described below.

The DNA pieces were first inserted into the mammalian expression vector based on the replicon of the Semliki Forest Virus (pSFV1). This expression system does not lead to a stable expression mammalian cell line but, however gives an indication whether or not the chimeric protein is expressed and whether the product is effectively secreted in the culture medium, which is advantageous for the purification procedure.

Stable expression in the culture medium of mammalian cell lines is preferred to obtain good quality and quantities of paramyxovirus glycoproteins. All the chimeric modules have been inserted in the shuttle vector, the pEE14, which integrates in the genome of mammalian cells such as CHO-K1. A quite good expression level was obtained with the RSV FxG homochimeric recombinant protein, however negligible expression was obtained for the FxHN recombinant homochimeric protein of either PIV3 or MuV. Expression of heterochimeric proteins was obtained from CHO cells.

Thus by constructing heterochimeric DNA molecules combining the extracellular domains of the F protein of one virus linked to the extra cellular domain of the HN or G protein of another virus and inserting them into the pEE14 vector for CHO expression it has been possible to raise the expression level of these proteins. These proteins may be used to achieve protection against at least two paramyxoviridae viruses with a single immunogen.

Some of the chimeric molecules have been inserted into the shuttle vectors, pAcUW51 and pACGP67, which integrate in the genome of bacterial and lepidopteran cells. Surprisingly good expression of heterochimeric proteins was obtained from insect cells.

Vector construction

Preliminary Constructs

a) Plasmid pNIV2819

Starting from plasmid pNIV2801, a cDNA clone encoding *inter alia* the F protein of RSV (type RSS-2; received from Dr Pringle, UK) we reconstructed a cDNA module coding for the F protein lacking the membrane anchor sequence.

Plasmid pNIV2801 was digested with *PstI* in order to recover a 1416 bp DNA piece encoding amino acid residues 18 to 489 of the F protein. Synthetic oligonucleotides, specifying respectively the sequences for amino acids 1 to 17 and 490 to 526, were used to produce the corresponding cDNA fragments by the polymerase chain reaction performed with pNIV2801 DNA as template. The primers were designed to generate also unique flanking restriction sites useful for subsequent cloning steps. The coding module was assembled, by ligation, from the three DNA pieces described above and introduced into the standard cloning vector pUC19, to create plasmid pNIV2819. This plasmid encodes the RSV F protein carrying its signal sequence but lacking its anchor sequence (figure 1).

b) Plasmid pNIV 2820

The cDNA module encoding the full length F protein of RSV was constructed as follows. Using two synthetic oligonucleotides, the polymerase chain reaction was performed with pNIV2801 DNA as template to generate a 273 bp DNA fragment

encompassing the sequence coding for aa 490 to aa 574 of the F protein, the stop codon and unique restriction sites useful for subsequent cloning steps. This fragment was digested with *Nsi*I and *EcoR*I and substituted for the *Nsi*I-*EcoR*I DNA piece present in the coding module of pNIV2819 (figure 2). The resulting plasmid, pNIV2820, thus encodes the RSV F protein carrying both signal and membrane anchor sequences.

c) Plasmid pNIV2841

In this construction, the DNA coding for aa 165 to 176 of the G protein of RSV is fused to the DNA encoding the RSV $Fs^{+}a^{-}$ protein. This part of the G protein is conserved among both subgroups of RSV.

The starting material, pNIV2819, was digested by *Nco*I and *Sma*I yielding a 1601 bp fragment. This fragment was subcloned into the *Nco*I and *Msc*I sites of pNIV103 (a derivative of pULB1221, see European Patent Application No. 186643) leading to pNIV2844. This subcloning allowed to place the translation initiation site of the F protein in a more favourable context according to the model proposed by Kozak (Kozak M, Nature 308, 241-246, 1984).

A 1605 bp fragment was recovered from pNIV2844 by digestion with *Kpn*I and *Sal*I and introduced by ligation into pUC19 digested with *Kpn*I and *Sal*I, creating pNIV2840.

Two complementary synthetic oligonucleotides specifying the sequence for amino acids 165 to 176 of the G protein followed by a stop codon and flanked by *Nsi*I, *BamH*i, *EcoR*I and *Hind*III sites were hybridized. The 55 bp resulting fragment was cloned into the pNIV2840 digested by *Nsi*I and *Hind*III, thus replacing a 142 bp DNA sequence encoding amino acids 491 to 526 of the F protein. The resulting recombinant plasmid, pNIV2841, thus contains the sequence coding for amino acids 1 to 490 of the F protein followed by amino acids 165 to 176 of the G protein (figure 3).

Vector Construction

I) For transfer into the pSFV1 vector

a) The RSV fusion protein lacking the membrane anchor domain fused to the MuV hemagglutinin-neuraminidase lacking the signal-anchor domain, F_{RSV} (1-526) HN_{MuV} (60-582).

Plasmid pNIV2875, a derivative of pNIV2820 which carries the DNA coding for the F protein of RSV in which the *Spe*I restriction site has been eliminated by site-directed mutagenesis into the pUC19 vector, has been digested by *Hind*III and *Bsp*HI, and a 1618 bp fragment has been isolated. Plasmid pNIV3229, a derivative of pNIV3215 whose construction has been already described in WO9425600 and which carries the DNA coding for the HN protein of MuV into the pUC19 vector, has been digested with *Bbs*1 and *Bam*HI; a 1580 bp fragment has been isolated. Both fragments were linked together by two complementary synthetic *Bsp*HI-*Bbs*1 oligonucleotides (Fig 4A) restoring the coding sequence of the chimeric molecule and were inserted into the *Bam*HI-*Hind*III site of the pUC19 vector leading to pNIV4102. (Fig4B) After the sequencing of the junction regions, the chimeric cassette was retrieved from pNIV4102 by a *Bam*HI digestion and was inserted into the *Bam*HI site of the pSFV1 vector (Liljeström, P. and Garoff,H. (1991) Bio/Technology 9, 1356). The resulting plasmid, pNIV4104, contains into the pSFV1 vector the sequence coding for amino acids 1 to 526 of the RSV F protein followed by amino acids 60 to 582 of the MuV HN protein. (Fig4C)

b) The RSV fusion protein lacking the membrane anchor domain fused to the PIV3 hemagglutinin-neuraminidase lacking the signal-anchor domain, F_{RSV} (1-526) HN_{PIV3} (70-572).

Plasmid pIBI-HN , a cDNA clone containing the complete coding sequence of protein HN of PIV3 as well as its 3' non coding sequence (received from Dr.K. Dimock, University of Ottawa, Canada), has been digested by *Ase*I and *Bam*HI and

a 1468 bp fragment has been isolated. Plasmid pNIV2875 (see supra), which carries the DNA encoding the F protein of RSV, in which the unique *Spe*I site has been eliminated by site-directed mutagenesis, inserted into the pUC19 vector, has been digested by *Bam*HI and *Bsp*HI, and a 1588 bp fragment has been isolated. Both fragments were linked together by two complementary synthetic *Bsp*HI-*Ase*I oligonucleotides (Fig5A) and were inserted into the *Bam*HI site of the pUC19 vector leading to pNIV4105 or to pNIV4109 (Fig5B) depending of the orientation of the chimeric module in the vector. After the sequencing of the junction region, the chimeric cassette was retrieved by a *Bam*HI digestion from pNIV4109 and inserted into the *Bam*HI site of the pSFV1 vector. The resulting plasmid, pNIV4110, contains, inserted into the pSFV1 vector, the sequence coding for amino acids 1 to 526 of the RSV F protein followed by amino acids 70 to 572 of the PIV3 HN protein. (Fig5C)

c) The PIV3 fusion protein lacking the membrane anchor domain fused to the RSV attachment protein lacking the signal-anchor domain, F_{PIV3} (1-492) G_{RSV} (69-298).

Plasmid pNIV3310, described in WO9306218 which carries the DNA coding for amino acids 1 to 484 of the PIV3 F protein followed by amino acids 87 to 572 of the PIV3 HN protein into the pIBI vector, was digested by *Eco*RI and *Bgl*II, and a 1435 bp fragment has been isolated. Plasmid pNIV2850, which carries the RSV G protein into the pUC19 vector, has been digested by *Mae*III and *Hind*III, and a 694 bp fragment has been isolated. Both fragments were then linked together by using two complementary *Bgl*II-*Mae*III synthetic linkers (Fig6A) and were inserted into the *Eco*RI-*Hind*III sites of pUC19 vector leading to pNIV4103 (Fig6B). The chimeric module was then retrieved from the pUC19 vector by a *Bam*HI-*Hind*III digestion. After treating the protruding ends with the Klenow polymerase, the chimeric cassette has been inserted into the *Sma*I site of pSFV1 vector. The resulting plasmid pNIV4106, thus contains the sequence coding for amino acids 1 to 492 of the F protein of PIV3 followed by amino acids 69 to 298 of the G protein of RSV inserted into the pSFV1 vector (Fig6C).

d) The PIV3 fusion protein lacking the membrane anchor domain linked to the MuV hemagglutinin-neuraminidase lacking the signal-anchor domain, F_{PIV3} (1-493) HN_{MuV} (60-582).

Plasmid pNIV3310 (see supra, FHN_{PIV3} in pIBI) was digested by *Eco*RI and *Bgl*II and a 1435 bp fragment was isolated. Plasmid pNIV3229 (see supra, HN_{MuV} into pUC19) was digested by *Bbs*I and *Hind*III, and a 1610 bp fragment was isolated. Both fragments were linked together by adding two synthetic complementary linkers specifying a *Bgl*II and a *Bbs*I ends (Fig7A) into the pUC19 vector leading to pNIV4117 (Fig7B). After sequencing the junction region, the chimeric cassette was retrieved from the pUC19 vector by a *Bam*HI digestion and was inserted into the *Bam*HI site of the pSFV1 vector. The resulting plasmid pNIV4118 encodes, cloned in the pSFV1 vector, the DNA sequence specifying amino acids 1 to 493 of the PIV3 fusion protein linked to amino acids 60 to 582 of the MuV HN protein (Fig7C).

e) The MuV fusion protein lacking its membrane anchor domain linked to the RSV attachment protein lacking its signal-anchor domain, F_{MuV} (1-482) G_{RSV} (69-298).

Plasmid pNIV3221, described in WO9425600 which carries the sequence encoding amino acids 1 to 462 of the MuV fusion protein within the pUC19 vector, has been digested with *Eco*RI and *Bsr*FI, and a 771 bp fragment has been purified. Plasmid pNIV3221 has been also digested with *Bsr*FI and *Pst*I, and a 628 bp fragment has been isolated. Plasmid pNIV2850 (see supra, G_{RSV} into the pUC19) has been digested with Mae*III and* Hind*III* and a 694 bp fragment has been isolated. The three fragments were linked together; the F_{MuV}/G_{RSV} junction was created by adding to the ligation reaction two synthetic complementary oligonucleotide specifying *Pst*I and *Mae*III sites (Fig8A), and were inserted into the *Eco*RI-*Hind*III sites of the pBluescript vector leading to pNIV4113(Fig8B). The chimeric cassette was recovered from pNIV4113 by a *Asp718*I digestion and, after treating the protruding

ends with the Klenow polymerase, was inserted into the *Sma*I site of the pSFV1 vector. The resulting plasmid, pNIV4114 contains into the pSFV1 vector the sequence specifying amino acids 1 to 482 of the MuV F protein linked to amino acids 69 to 298 of the RSV G protein (Fig8C).

f) The MuV fusion protein lacking its membrane anchor domain linked to the PIV3 hemagglutinin-neuraminidase lacking its signal-anchor domain, F_{MuV} (1-482) HN_{PIV3} (54-572).

Plasmid pNIV4113 (see supra, F_{MuV} x G_{RSV} in pBluescript) was digested by *Bsa*I and *Bam*HI, a 1469 bp fragment was isolated. Plasmid pNIV3308, described in WO9306218 and which carries the DNA sequence specifying amino acids 1 to 31 followed by amino acids 54 to 572 of the PIV3 HN protein into the pIBI vector, was digested by *Eco*RI and *Bam*HI and a 1569 bp fragment was isolated. Both fragments were linked together by two synthetic complementary linkers specifying *Bsa*I and *Eco*RI sites (Fig9A) into the *Bam*HI site of pBluescript leading to pNIV4115 (Fig9B). The chimeric module was recovered from pNIV4115 by a *Bam*HI digestion and was inserted into *Bam*HI site of pSFV1 vector. The resulting plasmid, pNIV4116, encodes, in the pSFV1 vector, the sequence specifying amino acids 1-482 of the MuV F protein fused to amino acids 54 to 572 of the PIV3 HN protein (Fig9C).

g) The RSV fusion protein lacking its membrane anchor domain linked to the RSV attachment protein lacking its signal-anchor domain, F_{RSV} (1-526) G_{RSV}(69-298).

Plasmid pNIV2857 (Fig16A), a derivative of pNIV2841 and which contains the DNA sequence coding for amino acids 1 to 526 of the RSV fusion protein linked to amino acids 69 to 298 of the RSV attachment protein, has been digested by *Asp718I* and *HindIII* and a 2180 bp fragment has been isolated. After treating the protruding extremities with Klenow's polymerase, this fragment has been inserted in the *Sma*I site of the pSFV1 vector. The resulting plasmid pNIV2870, contains in the pSFV1

vector, the DNA sequence coding for amino acids 1 to 526 of the RSV fusion protein linked to amino acids 69 to 298 of the RSV attachment protein (Fig16B).

II) For transfection into CHO cells

a) The RSV fusion protein lacking the membrane anchor domain fused to the MuV hemagglutinin-neuraminidase lacking the signal-anchor domain, F_{RSV} (1-526) HN_{MuV} (60-582).

Plasmid pNIV4102, (Fig10A, see supra, F_{RSV} x HN_{MuV} into the pUC19 vector) has been digested with *Bam*HI, and after treating the protruding ends with the Klenow polymerase, the chimeric module has been inserted into the *Sma*I site of the glutamine synthetase (GS) vector, pEE14 (Cockett *et al*, 1990, Bio/Technology 8, 662-667). The resulting plasmid pEE14 Fs^+a^- RSV x HN s^-a^- MuV contains sequences coding for amino acids 1 to 526 of the RSV F protein fused to amino acids 60 to 582 of the MuV HN protein under the control of the major immediate early promoter of the human cytomegalovirus (hCMV-MIE) (Fig10B).

b) The RSV fusion protein lacking its membrane anchor domain linked to the PIV3 hemagglutinin-neuraminidase lacking its signal-anchor domain, F_{RSV} (1-526) HN_{PIV3} (70-572).

Plasmids pNIV4105 and pNIV4109 (Fig11A and B, see supra, F_{RSV} x HN_{PIV3} into the pUC19 vector) were digested by *Eco*RI and *Xho*I and a 2032 bp as well as a 1064 bp fragments were isolated. Both fragments were inserted together into the *Eco*RI site of pEE14. The resulting plasmid pEE14 Fs^+a^- RSV x HNs^-a^- PIV3 contains sequences coding for amino acids 1 to 526 of the RSV F protein fused to amino acids 70 to 572 of the PIV3 HN protein under the control of the hCMV promoter (Fig11C).

c) The PIV3 fusion protein lacking the membrane anchor region linked to the RSV attachment protein lacking the signal-anchor domain, F_{PIV3} (1-492) G_{RSV} (69-298).

Plasmid pNIV4103 (Fig12A, see supra, F_{PIV3} x G_{RSV} into the pUC19 vector) was digested by *Hind*III and a 2180 bp fragment was isolated. After treating the protruding extremities with the Klenow polymerase, the chimeric module was inserted into the *Sma*I site of the pEE14 vector. The resulting plasmid, pEE14 Fs^+a^- PIV3 x Gs^-a^- RSV, contains, under the control of the hCMV promoter, the sequence encoding amino acids 1 to 492 of the PIV3 F protein followed by amino acids 69 to 298 of the RSV G protein (Fig 12B).

d) The PIV3 fusion protein lacking the membrane anchor domain fused to the MuV hemagglutinin-neuraminidase lacking the signal-anchor domain, F_{PIV3} (1-493) HN_{MuV} (60-582).

Plasmid pNIV4117 (Fig13A, see supra, F_{PIV3} HN_{MuV} into the pUC19 vector) was digested with *Hind*III and a 3119 bp fragment was isolated and inserted into the *Hind*III site of the pEE14 vector. The resulting plasmid, pEE14 Fs^+a^- PIV3 x HNs^-a^- MuV, contains under the control of the hCMV promoter a sequence encoding amino acids 1 to 493 of the PIV3 fusion protein fused to amino acids 60 to 582 of the MuV HN protein (Fig13B).

e) The MuV fusion protein lacking its membrane anchor domain fused to the RSV attachment protein lacking its signal-anchor domain, F_{MuV} (1-482) G_{RSV} (69-298).

Plasmid pNIV4113 (Fig14A, see supra, F_{MuV} G_{RSV} into the pBluescript vector) has been digested *Asp718*I, the protruding ends have been treated by the Klenow polymerase. A 2200 bp fragment has been isolated and inserted into the *Sma*I site of pEE14. The resulting plasmid, pEE14 Fs^+a^- MuV x Gs^-a^- RSV, has, under the

control of the hCMV promoter, the sequence encoding amino acids 1 to 482 of the MuV F protein followed by amino acids 69 to 298 of the RSV G protein (Fig14B).

f) The MuV fusion protein lacking its membrane anchor domain fused to the PIV3 hemagglutinin-neuraminidase lacking its signal-anchor domain, F_{MuV} (1-482) HN_{PIV3} (54-572).

Plasmid pNIV4115 (Fig15A, see supra, F_{MuV} x HN_{PIV3} into the pBluescript vector) has been digested with *Eco*RI and a 3040 bp fragment has been inserted into the *Eco*RI site of the pEE14 vector. The resulting plasmid, pEE14 Fs^+a^- MuV x HNs^-a^- PIV3, contains, downstream to the hCMV promoter region, a sequence coding for amino acids 1 to 482 of the MuV F protein followed by amino acids 54 to 572 of the PIV3 HN protein (Fig15B).

g) The RSV fusion protein lacking its membrane anchor domain linked to the RSV attachment protein lacking its signal-anchor domain, F_{RSV} (1-526) G_{RSV}(69-298).

Plasmid pNIV2857 (Fig17A), a derivative of pNIV2841 and which contains the DNA sequence coding for amino acids 1 to 526 of the RSV fusion protein linked to amino acids 69 to 298 of the RSV attachment protein, has been digested by *Asp718I* and *HindIII* and a 2180 bp fragment has been isolated. After treating the protruding extremities with Klenow's polymerase, this fragment has been inserted the *SmaI* site of the pEE14vector. The resulting plasmid, pEE14 Fs^+a^- RSV x Gs^-a^- RSV, contains under the control of the hCMV promoter the DNA sequence coding for amino acids 1 to 526 of the RSV fusion protein linked to amino acids 69 to 298 of the RSV attachment protein (Fig17B).

h) The original RSV fusion protein lacking the membrane anchor domain linked to the PIV3 hemagglutin-neuraminidase lacking the signal-anchor domain, F_{RSV} (1-526) HN_{PIV3} (70-572) bis.

Plasmid pNIV2852, a derivative of pNIV2820 which carries the DNA encoding the RSV F protein where the translation initiation site is in a more favourable context according to the model proposed by Kozak (Kozak M, Nature 308, 241-246, 1984), has been digested *BamHI* and *BspHI*, and a 1588 bp fragment has been isolated.

Plasmid pIBI-HN, a cDNA clone containing the complete coding sequence of the HN protein of PIV3 (received from Dr. K. Dimock, University of Ottawa, Canada) has been digested by *AseI* and *BamHI* and a 1468 bp has been isolated.

Both fragments were linked together by two complementary synthetic *BspHI-AseI* adaptators (Fig18A) and were inserted into the BamHI site of the pUC19 vector leading to pNIV4120 (Fig18B).

After the sequencing of the junction region, the chimeric cassette was retrieved by a *BamHI* digestion from pNIV4120 and inserted into the *BamHI* compatible *BclI* site of the pEE14 vector. The resulting plasmid pEE14 Fs^+a^-RSV x HNs^-a^- PIV3 bis contains the sequences coding for amino acids 1 to 526 of the RSV F protein fused to amino acids 70 to 572 of the PIV3 HN protein under the control of the hCMV promoter (Fig18C).

This construct differs from the earlier pEE14 Fs^+a^-RSV x HNs^-a^- PIV3 construct (II-a) in the F coding region. In $F_{RSV}HN_{PIV3}$ **bis,** the nucleic acid sequence found in $F_{RSV}HN_{PIV3}$, ATG GAT CTG (those codons are specifying aa Met1, **Asp2** and Leu3) and ACC AGT (specifying aa Thr54 and Ser 55) is replaced by the original sequence of the RSV F protein that is ATG GAG TTG (specifiyng aa Met1, **Glu2**, Leu3) and ACT AGT (specifying Thr54 and Ser55).

i) The original RSV fusion protein lacking the membrane anchor domain linked to the PIV3 hemagglutinin-neuraminidase lacking the signal-anchor domain with, at the C-terminal part, a polyhistidine tail preceded by the enterokinase cleavage site, F_{RSV} (1-526) HN_{PIV3} (70-572) en his

Plasmid pIBI-HN, a cDNA containing the PIV3 HN protein coding sequence (see supra) has been digested by *PstI* and *SphI*. A 4588 bp fragment has been isolated and linked to complementary synthetic *PstI-SphI* adaptators (Fig19A).

After the sequencing of the junctions as well as the synthetic linkers, the resulting plasmid pNIV3340 has been digested by *XhoI* and *BamHI* and a 1121 bp fragment has been isolated (Fig19B).

Plasmid pNIV4120 (see supra) has been digested by *XhoI* and *BamHI* and a 2017 bp fragment has been isolated (Fig19C).

Both fragments were linked together and inserted into the *BamHI* compatible *BclI* site of the pEE14 vector. The resulting plasmid pEE14 FRSVs^{+}a^{-} x HNs^{-}a^{-} en his contains, under the control of the hCMV promoter, sequences coding for amino acids 1 to 526 of the RSV fusion protein fused to the amino acids 70-572 of the PIV3 HN protein fused to the enterokinase cleavage site,({Asp} x4 Lys) followed by a polyhistidine tail ({his}x6) and a stop codon (Fig19D).

j) The signal domain of the tissue plasminogen activator fused to the yeast ubiquitin followed by the enterokinase cleavage recognition site and the original RSV fusion protein lacking its membrane signal and anchor domains linked to the PIV3 hemagglutin-neuraminidase lacking the signal-anchor domain, sTPA(1-21) UB(1-74) ent F_{RSV} (24-526) HN_{PIV3} (70-572)bis.

1) The signal domain of the tissue plasminogen activator fused to the yeast ubiquitin.

A 208 bp fragment corresponding to amino acid 1 to 76 of the ubiquitin protein of *Saccharomyces cerevisiae* was isolated by a digestion of pNIV3475 (a derivative of YEPUBSTUALL, a yeast 2 μ vector backbone carrying the yeast ubiquitin) with *BamHI* and *XbaI* (Fig 20A).

Plasmid JW4304 (received from J. Mullins, University of Washington, U.S.A) which encodes the signal domain of the tissue plasminogen activator (sTPA) was digested by *NheI* and *BamHI* and a 5115bp was isolated. Both fragments were linked together using two synthetic complementary *NheI-XbaI* adaptators (Fig20B). The resulting plasmid pNIV4121 was digested by *HindIII* and *BamHI*. A 330 bp fragment was isolated and inserted into the *HindIII* and *BamHI* sites of the pBluescript vector. The resulting plasmid pNIV4122 contains the DNA sequence specifying the signal domain of the tissue plasminogen activator followed by an alanine and a serine residue (those two amino acids are known to produce a good leader cleavage) fused to the yeast ubiquitin (Fig 20C).

2) The signal domain of the tissue plasminogen activator linked to the yeast ubiquitin followed by the enterokinase cleavage recognition site and amino acid 24 to 55 of the original fusion protein of RSV.

Plasmid pNIV4122 (Fig 21A, see supra) was digested by *AflII* and *SpeI*. A 3212 bp fragment was isolated and linked to synthetic complementary *AflII-SpeI* adaptators (Fig21B). The entire module was then sequenced. The resulting plasmid pNIV4123 encodes the signal domain of the tissue plasminogen activator linked to the N-terminal 74 aa of the yeast ubiquitin followed by the recognition site of enterokinase {(Asp)4 Lys} and amino acid 24 to 55 of the original fusion protein of RSV (Fig21C).

3) The signal domain of the tissue plasminogen activator linked to the yeast ubiquitin followed by the enterokinase cleavage recognition site and the RSV fusion protein linked to the PIV3 hemagglutin-neuraminidase lacking their membrane domains.

Plasmid pNIV4123 (Fig 22A, see supra) was digested by *HindIII*, treated by the Klenow polymerase and digested by *SpeI*. A 408 bp fragment has been isolated.

Plasmid pNIV4120 (Fig 22B, see supra) has been digested by *XbaI*, treated by the Klenow polymerase, and digested by *SpeI*. A 5620 bp fragment has been isolated.

Both fragment have been linked together to generate pNIV4124 (Fig 22C).

The entire coding module was retrieved from pNIV4124 by a digestion with *XbaI* and *EcoRI* and was inserted into the *XbaI* and *EcoRI* sites of the pEE14 expression vector. The resulting plasmid pEE14 sTPA x UBI x EN x Fs-a-RSV x HNs-a-PIV3, contains, under the control of the hCMV promoter, the sequence coding for aa1-21 of the tissue plasminogen activator followed by an alanine and a serine residue, by the 74 N-terminal amino acids of the yeast ubiquitin, by the recognition cleavage site of the enterokinase ({Asp}4 Lys), by aa 24-526 of the original RSV fusion protein and by aa 70-572 of the hemagglutin-neuraminidase of PIV3.

III) For transfection into Insect Cells

a) The original RSV fusion protein lacking the membrane anchor domain linked to the PiV3 hemagglutin-neuraminidase lacking the signal-anchor domain, F_{RSV} (1-526) HN_{PIV3} (70-572) bis.

Plasmid pNIV4120 (FIG 23A) was digested by *BamHI* and a 3114 bp fragment was isolated and inserted into the *BamHI* site of the baculovirus transfer vector, pAcUW51 (PharMingen). The resulting plasmid pNIV4132 (Fig 23B) contains, under the control of the polyhedrin promoter, the sequence coding for amino acids 1-526 of the RSV F protein fused to amino acids 70-572 of the PiV3 HN protein.

b) The baculovirus gp67 signal peptide fused to the original RSV fusion protein lacking both membrane signal and anchor domain linked to the PiV3 hemagglutin-neuraminidase lacking the signal-anchor domain, $sGP67F_{RSV}$ (25-526) HN_{PIV3} (70-572) bis.

Plasmid pNIV4120 (FIG 24A, see supra) was digested by *BamHI* and *SpeI* and a 2939 bp fragment was isolated, linked to two complementary synthetic *BamHI-SpeI* adaptators and inserted into the *BamHI* site of the baculovirus transfer vector, pAcGP67A (PharMingen). The resulting plasmid pNIV4136 (Fig 24) contains, under the control of the polyhedrin promoter, the sequence coding for amino acids 1-38 of the Baculovirus gp67 protein, followed by an Alanine and an Aspartate linked to amino acids 25-526 of the RSV F protein fused to amino acids 70-572 of the PiV3 HN protein.

Expression in eukaryotic cells

A) via the pSFV1 vector

The pSFV1 vector is based on the Semliki Forest Virus (SFV) replicon. The DNA of interest is cloned into the pSFV1 vector that serves as a template for *in vitro* synthesis of recombinant RNA. The RNA is transfected into mammalian cells such as BHK-21 cells. The recombinant RNA in the cells drives its own replication and capping resulting in production of heterologous protein.

Plasmids pNIV2870 was digested with *PvuI*; pNIV4106, pNIV4110, pNIV4114, pNIV4116 and pNIV4118 were digested with *SpeI* prior to RNA transcription. After a phenol extraction followed by an ethanol precipitation, 2 μg of linearized DNA was used as a template for RNA production. About 5 μg RNA was used to transfect, by electroporation, about 8 10^6 BHK-21 cells. All experimental procedures for RNA production and cell transfection are detailed in Liljestrom and Garoff (Bio/Technology, 1991, 9, 1356).

After 24 h to 48 h post-electroporation, cells and spent culture medium have been collected for ELISA and radioimmunoprecipitation assays.

a) **pNIV4104, F_{RSV} HN_{MuV}**

ELISA were done using mAb 2072 anti-HN MuV (Örvell, 1984, *J. Immunology 132*, 2622-2629) or 20RG45, a goat anti-RSV serum (Fitzgerald, U.S.A.) to coat the microtiter plates and a rabbit polyclonal anti-SBL-1 (MuV) serum or mAb19 anti-F RSV (G.Taylor, Inst. of Animal Health, Compton Lab., U.K.) as capture antibody.

Radioimmunoprecipitation of the ^{35}S-methionine labelled product was done using mAb2072 (Örvell) and products were resolved onto 7.5% SDS-PAGE.

b) pNIV4110, F_{RSV} HN_{PIV3}

ELISA were done using anti-RSV goat serum 20RG45 or mAb anti-HN_{PIV3} 4830 (Rydbeck *et al*, *J. Gen. Virol. 67*, 1531-1542, 1986) to coat microtiter plates and mAb19 anti-F RSV (G.Taylor) or rabbit anti-PIV3 (E.Norrby, Stockholm) serum as a capture antibody.

Radioimmunoprecipitation was done using anti-HN PIV3 mAb4830.

c) pNIV4106, F_{PIV3} G_{RSV}

ELISA were done using mAb anti-F_{PIV3} 4549 (E.Norrby, Stockholm) or mAb anti G_{RSV} 858-2 (Chemicon, U.S.A.) to coat microtiter plates and a rabbit anti-PIV3 serum as a capture antibody.

Radioimmunoprecipitation was done using mAb anti-F_{PIV3} 3283 (Behringwerke).

d) pNIV4118, F_{PIV3} HN_{MuV}

ELISA plates were coated with anti-F PIV3 mAb 1031215 (Norrby) or with mAb 2072 anti-HN MuV (Örvel) and rabbit anti-PIV3 sera or rabbit anti-MuV sera were used as capture antibody.

Immunoprecipitation of labelled product was done using mAb 2072 anti-HN MuV.

e) pNIV4114, F_{MuV} x G_{RSV}

ELISA plates were coated with anti-F MuV monoclonal 5414 (Örvell) or anti G_{RSV} mAb (Chemicon) and a rabbit anti-SBL-1 serum was used as a capture antibody.

f) pNIV4116, F_{MuV} x HN_{PIV3}

ELISA plates were coated with anti-F MuV mAb 5414 (Örvell) or mAb anti-HN PIV3 4830 (Norrby) and rabbit anti-SBL-1 serum or a rabbit anti-PIV3 serum as a capture antibody.

g) pNIV2870, F_{RSV}x G_{RSV}

ELISA were done using 20RG45, a goat anti-RSV serum (Fitzgerald, U.S.A.) to coat the microtiter plates and mAb19 anti-F RSV (G.Taylor, Inst. of Animal Health, Compton Lab., U.K.) as capture antibody.

B) <u>Expression in CHO cells (stable transformants)</u>

All recombinant plasmids were transfected by calcium phosphate coprecipitation into CHO-KI cells, using 20 μg DNA per 1.25 10^6 cells. The CHO-KI cells were grown in GMEM-S medium. The GS transfectants were selected by adding 25 μM methionine sulfoximine to the culture medium two days after transfection. After ten to fourteen days, resistant colonies were picked and transferred into 96 wells plates. Each transformant was then transferred into 24 wells plates and subsequently to 80 cm^2 flasks. The GS transformants were assayed for the recombinant products when cells reached about 80% confluency. The procedure follows the one described in Cockett *et al* (Bio/Technology, 1990, *8*, 662-667).

ELISA and immunoprecipitation of radiolabelled products were done using the same procedures as the ones described above for the pSFV1 system.

Results

	PSFV1		CHO			
Chimeric products	Expression	Secretion	Expression	Secretion	Size (kDa)	
F_{RSV} G_{RSV}	+	+	+	+	±130	homochimeric products
F_{PIV3} HN_{PIV3}	+	-	undetectable			
F_{MuV} HN_{MuV}	+	-	undetectable			
F_{RSV} HN_{MuV}	+	+	+	+	± 135	heterochimeric products
F_{RSV} HN_{PIV3}	+	+	+	+	± 130	
F_{PIV3} G_{RSV}	+	+	+	+	± 130	
F_{PIV3} HN_{MuV}	+	+	+	+	± 130	
F_{MuV} G_{RSV}	+	+	+	+	± 130	
F_{MuV} HN_{PIV3}	+	+	+	+	± 120	

Expression in Insect cells

a) **Expression in lepidopteran cells.**

The vector pAcUW51 is a shuttle vector for bacteria and lepidopteran cells. A heterologous protein coding sequence can be inserted downstream the baculovirus p10 promoter or either downstream the polyhedrin promoter.

The pAcGP67 vector is a shuttle vector for bacteria and lepidopteran cells that contains the gp67 signal sequence upstream a multiple cloning site. A heterologous gene can be inserted in one of the cloning site and will be expressed as a gp67

signal peptide fusion protein under the control of the polyhedrin promoter. The gp67 signal peptide mediates the secretion of the recombinant protein.

Either pAcUW51 or pAcGP67 recombinant plasmid can be transfected along with baculovirus linearised DNA into Sf9 cells (Baculogold DNA, PharMingen). This leads to the generation of a recombinant baculovirus stock. The expression of the recombinant heterologous protein is obtained by infecting insect cells with the recombinant baculovirus

Plasmid pNIV4132 or plasmid pNIV4136 were transfected with baculovirus linearised DNA into Sf9 cells. Recombinant baculovirus 3546 (derived from cells transfected by pNIV4132) or 5V (derived from cells transfected by pNIV4136) were plaque purified and were used to infect Sf9 or High Five™ cells (Invitrogen). 24h to 72 h post-infection the cells and the spent culture medium have been collected for ELISA and Western blot analysis.

ELISA were done using anti-RSV goat serum 20RG45 (Fizgerald) to coat microtiter plates and mAb19 anti-F RSV (G.Taylor) as a capture antibody.

Western blots were done using mAb19 anti-F RSV (G.Taylor) or using anti-RSV goat polyclonal serum 20RG45 (Fizgerald).

The spent medium from cells infected by either baculovirus 3546 or by 5V tested positive in ELISA. The level of expression, depending on the host cell line (SF9 or High Five), multiplicity of infection, medium (fetal calf serum supplemented or serum free synthetic medium) was at least ten times higher than the one obtained with a recombinant CHO-KI clone obtained by transfection with pEE14 F_{RSV} (1-526) HN_{PiV3} (70-572)bis .

In addition, the spent medium of the baculovirus infected cells reacted positively in Western blot. A band in the vicinity of 110kDa was present in the immunoblots. These

results confirm the secretion of the chimeric F_{RSV}-HN_{PiV3} into the medium of Sf9 and High Five cells infected with the recombinant baculoviruses.

b) Purification of the recombinant product

SF9 cells, adapted to serum free medium, were infected with the plaque purified recombinant baculovirus V5 or 3546. The cells were grown in suspension in 500ml Erlenmeyer flask in SF900II medium (Gibco BRL). The medium from virus infected cells were harvested two days post-infection. The soluble F_{RSV}-HN_{PiV3} product was purified from the medium of infected cells by immunoaffinity chromatography using an anti-F RSV monoclonal antibody, mAb19. The anti-F monoclonal antibody was coupled to Activated CH Sepharose 4B (Pharmacia) following the manufacturer instructions. The immunoaffinity gel was washed 3 times with 10 bed volumes of buffer A (20mM phosphate buffer pH 6.4, NaCl 150mM) prior to sample loading. After 16 hours at 4°C, the gel was washed with buffer A and the chimeric product was eluted with 100mM phosphoric acid. Eluted protein was neutralized immediately with one tenth of volume of 1M phosphate buffer pH 7.

SDS-PAGE of the immunoaffinity-purified F_{RSV}-HN_{PiV3} revealed the presence of a major protein band of about 110 kDa. This protein was visualized by Coomassie blue staining of the gel and reacted with the monoclonal antibody anti-F_{RSV} (mAb19) or with the polyclonal serum (20RG45) on immunoblots (Fig25).

c) production of polyclonal antibodies

In order to obtain specific antibodies, the baculovirus derived F_{RSV}-HN_{PiV3} protein, purified by immunoaffinity as described above, was used to immunise four BalbC mice and two New Zealand white rabbits. Three sub-cutaneous injections of 20µg/ml/dose/rabbit or 6µg/100µl/dose/mouse were done at three weeks interval. The sera were collected 3 weeks after the second and the third injection and the antibody response was detected using ELISA and Western blots assays.

1) ELISA assays

a) Mice response

The antibody response was followed using a goat anti-RSV serum (2ORG45, Fitzgerald, USA) to coat the microtiter plates and mouse anti-FHN sera as capture antibody. The antigens used were either the Fa_{RSV}-Drosophila or CHO derived, the F_{RSV}-HN_{PiV3} expressed in baculovirus and the medium of CHO cells transfected by the pEE14 was used as a negative control.

3 our of 4 mice sera collected after the second injection showed some but low specific response. However, the mice sera collected after the third injection showed a high increase in level of specific antibodies.

b) Rabbit response

The antibody response was followed using either one of the following ELISA. The antigens were the same as the one used to detect the mice antibody response.
Either a goat anti-RSV serum (2ORG45, Fitzgerald, USA), either a monoclonal antibody directed against the RSV fusion protein (mAb19, Compton Lab, UK) or a monoclonal antobody directed against the PiV3 hemagglutinin-neuraminidase (mAb3285, Behring) were used to coat the microtiter plate and the rabbit anti-HN sera was used as a capture antibody. The first and the second test bleeds generated high specific antibodies.

2) Western blot assays

Recombinant Fa-RSV CHO-KI ou Drosophila derived, F_{RSV}-HN_{PiV3} baculovirus derived or the CHO-pEE14 spent medium culture were electrophoresed onto a 15% SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham). The rabbit anti-HN sera as well as the mouse anti-HN sera detected specifically either the F protein or the F_{RSV}-HN_{PiV3} chimera.

Example 2

i) Optimization of the codon usage of the nucleic acids sequence coding for the RSV fusion protein lacking the membrane anchor domain linked to the PiV3 hemagglutin-neuraminidase lacking the signal-anchor domain, F_{RSV} (1-526) HN_{PiV3} (70-572) for the expression in mammalian cells.

A table showing the comparison of the codon usage found in the $F_{RSV}HN_{PiV3}$ module with the one found in highly expressed human gene can be found in Fig.26. As noted, the most prevalent codons found in the $F_{RSV}HN_{PiV3}$ module have an A or a T at their third degenerative position, whereas the human prevalent codons have a C or a G. For the improvement of the $F_{RSV}HN_{PiV3}$ protein expression, the entire coding sequence has been re-engineered to fit at best the human codon usage. The re-engineered sequence was obtained using synthetic long oligonucleotides, polymerase chain reaction (PCR) and conventional cloning procedures.

Re-engineering of the coding sequence of the $F_{RSV}HN_{PiV3}$ module

The entire synthetic sequence was recovered by joining three PCR fragments (A, B and C). The general strategy to obtain each PCR fragment is schematically represented in Fig 27. It consists of assembling overlapping long oligonucleotides in a first round amplification. The resulting full size fragment is further amplified using two short primers located on each of its extremities.

Construction of fragment A

The first PCR fragment, corresponding to 18 bases encoding restriction sites followed by bases 1 to 1269 of the $F_{RSV}HN_{PiV3}$ followed by 8 bases encoding restriction sites, was obtained by PCR assembly of 18 overlapping oligonucleotides (Fig 28). This fragment has been inserted in the pCRIITOPO cloning vector (Invitrogen). After sequencing the fragment, it was retrieved from the pCRIITOPO vector by a *XbaI* and *BsrGI* digestion and inserted into the corresponding sites of pNIV4120. The module corresponding to $F_{RSV}HN_{PiV3}$ with bases 1 to 1264 humanized was then retrieved by an *XbaI* and *EcoRI* digestion and inserted into the corresponding sites of pEE14 (Fig.29) generating pEE14xF_{RSV}humHN_{PiV3}.

Construction of fragment B

The second PCR fragment B corresponding to 13 bases encoding unique restriction sites followed by bases 1264 to 2136 of $F_{RSV}HN_{PiV3}$ was obtained by assembling 10 oligonucleotides whose sequences can be found in Fig.30. This fragment has been inserted in the pCRIITOPO vector and sequenced. This fragment has been recovered by a *BsrGI* and *KpnI* digestion.

Construction of fragment C

The third PCR fragment corresponding to bases 2023 to 3090 followed by 6 extra bases encoding an EcoRI site has been assembled starting from the 15 oligonucleotides shown in Fig 31. This fragment has been inserted in the pCRIITOPO cloning vector and sequenced. This fragment has been retrieved by a *KpnI* and *EcoRI* digestion (Fig 31).

Construction of the entire coding sequence

The entire $F_{RSV}HN_{PiV3}$ codon optimized coding sequence has been obtained by assembling fragment A, B, C as shown in Fig.32. pNIV4120 in which the PCR fragment A has replaced the original sequence (see Fig.29) was digested by *BsrGI* and *EcoRI*. The original sequence was eliminated and replaced by the *BsrGI- KpnI* fragment B and the *KpnI-EcoRI* fragment C. The codon optimized module was retrieved from the PCRIITOPO vector by a *XbaI* and an *EcoRI* and inserted in the corresponding sites of the pEE14 vector. The resulting plasmid, $pEE14F_{RSV}$ $humHN_{PiV3}hum$, encodes for the entire humanized coding sequence. The humanized $F_{RSV}HN_{PiV3}$ nucleic acids sequence is shown in Fig. 33.

Expression in CHO-KI cells

The recombinant pEE14 F_{RSV} $humHN_{PiV3}$ (see construction of fragment A, above, or recombinant $pEE14F_{RSV}$ $humHN_{PiV3}hum$ see construction of the entire coding sequence, above) was transfected using the FuGene reagent (Boeringer Mannheim), using 5 μg DNA per 1.25 10^6 cells. The CHO-KI cells were grown in GMEM-S medium. The GS transfectants were selected by adding 25 μM methionine sulfoximine to the culture medium two days after transfection. After ten to fourteen

days, resistant colonies were picked and transferred into 96 wells plates. Each transformant was then transferred into 24 wells plates and subsequently to 80 cm^2 flasks. The GS transformants were assayed for the recombinant product when cells reached about 80% confluency. The procedure follows the one described in Cockett *et al* (Bio/Technology, 1990, *8*, 662-667). Alternatively, the expression was evaluated three to five days after the addition of sodium butyrate (2mM) in the cell culture.

To compare the expression level to that of the non humanized $F_{RSV}HN_{PiV3}$, ELISA assays were done, using 20RG45, a goat anti-RSV serum (Fizgerald, U.S.A.) to coat the microtiter plates and mAb19 anti-F RSV (G. Taylor, Inst. of Animal Health, Compton Lab, U.K.) as capture antibody. The expression level was estimated using a purified $Fa\text{-}_{RSV}$ expressed in the Drosophila system.

The level of expression of the non-humanized expressed product by $pEE14F_{RSV}HN_{PiV3}$ didn't exceed 0.03 mg/L and 0.1 mg/L when sodium butyrate was added to the culture medium. The level of expression of the partially humanized product expressed by $pEE14F_{RSV}$ $humHN_{PiV3}$, reached 1 mg/L and up to 3 mg/L when sodium butyrate was added in the culture medium. The humanization of the sequence coding for amino acids 1-423 of the 1029 amino acids thus enhanced the level of expression up to 30 fold (see Figure 34a).

The level of expression of the entirely humanized product expressed by $pEE14F_{RSV}$ $humHN_{PiV3}hum$ was at least of 2 mg/L and reached up to 50 mg/L when sodium butyrate was added in the culture medium. The humanization of the entire coding region of $F_{RSV}HN_{PiV3}$ thus enhanced the level of expression of at least 200 to 500 fold (see Figure 34b).

ii) Optimization of the codon usage of the nucleic acids sequence coding for the mumps virus (MuV) fusion protein lacking the membrane anchor domain linked to the measles virus (MV) lacking the signal-anchor domain, F_{Muv} (1-482) H_{Mv} (59-617) for the expression in mammalian cells.

A table showing the comparison of the codon usage found in the $F_{MuV}H_{MV}$ module with the one found in highly expressed human gene can be found in Fig.35. As it can be seen, the codon usage frequencies of this chimerical gene is quite different from those prevalent in the human genome. For the improvement of the $F_{MuV}H_{MV}$ protein expression, the entire coding sequence has been re-engineered to fit at best the human codon usage. The re-engineered sequence was obtained using synthetic long oligonucleotides, polymerase chain reaction (PCR) and conventional cloning procedures.

Re-engineering of the coding sequence of the $F_{MuV}H_{MV}$ module

The entire synthetic sequence was recovered by joining four PCR fragments (A, B, C and D). The general strategy to obtain each PCR fragment is schematically represented in Fig 36. It consists of assembling overlapping long oligonucleotides in a first round amplification. The resulting full size fragment is further amplified using two short primers located on each of its extremities.

Construction of fragment A

The first PCR fragment, corresponding to 13 bases specifying restriction sites and a Kozak consensus motif followed by bases 1 to 1026 of the $F_{MuV}H_{MV}$ was obtained by PCR assembly of 12 overlapping oligonucleotides (Fig 37). This fragment has been inserted in the pCRIITOPO cloning vector (Invitrogen). After sequencing the fragment, it was retrieved from the pCRIITOPO vector by a *XbaI* and *TspRI* digestion and a 963 bp fragment was further purified, leading to fragment A.

Construction of fragment B

The second PCR fragment B corresponding to bases 965 to 1712 of $F_{MuV}H_{MV}$ was obtained by assembling 9 oligonucleotides whose sequences can be found in Fig.38. After its insertion into the pCRIITOPO vector and its sequencing, this 785 bp fragment has been recovered by a *TspRI* and *AvaI* digestion.

Construction of fragment C

The third PCR fragment C corresponding to bases 1712 to 2485 has been assembled starting from the 11 oligonucleotides shown in Fig 39. It has been inserted in the pCRIITOPO cloning vector and sequenced. This 774 bp fragment has been retrieved by an *AvaI* and *ApaI* digestion.

Construction of fragment D

The fourth PCR fragment D corresponding to bases 2485 to 3139 followed by 8 bp specifying a unique restriction site has been assembled starting from the 8 oligonucleotides shown in Fig 40. This fragment has been inserted in the pCRIITOPO vector and sequenced. A 657 bp fragment has been recovered after an *ApaI* and *EcoRI* digestion.

Construction of the entire coding sequence

The entire $F_{MuV}H_{Mv}$ codon optimised coding sequence has been obtained by assembling fragment A, B, C, D and inserting the module digested by *XbaI* and *EcoRI* into the corresponding sites of the pEE14 vector (Fig. 41). The resulting plasmid, pEE14F_{MuV}humH_{Mv}hum, encodes for a humanised sequence coding for aa 1-482 of the mumps virus fusion protein followed by aa 59-617 of the measles virus. The humanised and original $F_{MuV}H_{MV}$ nucleic and amino acids sequences are shown in Fig. 42.

iii) Purification and analysis of FHN expressed in CHO-KI

a) Purification

CHO cell line expressing secreted recombinant FHN was cultivated in cell factories in G-MEM medium supplemented with 2% FCS, in presence or absence of 1% Butyrate Na. FHN was purified by immunoaffinity chromatography by loading spent culture medium onto a Mab19-sepharose column as described using the same experimental conditions.

When expressed in absence of Butyrate Na, purified FHN migrated on SDS-PAGE, in heating and reducing conditions, mainly as a band of 110 kDa. In contrast, FHN is visualized as a triplet of 110, 120 and 130 kDa when CHO cells are cultivated with butyrate. Heating has a more drastic effect than reducer on the FHN electrophoretic migration. Indeed, high molecular weight species are clearly detected in the preparation when electrophoresis proceeded without heating suggesting the presence of FHN aggregates or oligomers. These aggregates did not seem to be contaminated by CHO proteins. Antibodies directed to CHO proteins did not specifically recognize on Western blot any bands. Glycan analysis was performed using several lectins specific for different carbohydrate moieties. Surprisingly, FHN did not carry sialic acids or high-mannose structures but carbohydrates of galactose-acetyl-galactosamine type characteristic of hybrid N- and/or O-glycosylations.

N-terminal microsequence analysis showed mainly the presence of F1 subunit in bands of 110-130kDa. The F2 N-terminal amino acid sequence detected in bands of lower and higher molecular weight indicated that some purified FHN molecules are present under a F0 form (non mature F).

The presence of aggregates or oligomers in the FHN preparations was confirmed by gel filtration analysis and proteins were detected by laser-light scattering. Whatever the culture conditions (butyrate or not), between 50 and 65% of FHN populations displayed a molecular weight higher than 10^6 Da demonstrating that FHN is aggregated. 5 to 15% has a molecular weight ranging from 400 to 900 kDa whereas 30 to 35% is monomeric FHN.

b) Serum immunoglobin analysis.

Immunisation protocol

The $F_{RSV}HN_{PiV3}$ protein was purified from the spent medium culture of the CHO-KI cells transfected by the recombinant pEE14 $F_{RSV}humHN_{PiV3}hum$ by immunoaffinity chromatography as described (Purification of the recombinant product expressed in baculovirus recombinant infected SF9 cells). The product was injected in 7 groups of Balb C1 mice as descibed in the following table 1.

Humoral response directed against the FHN protein

The humoral response directed against the FHN protein was determined. To this end, ELISA plates were coated with immunoaffinity purified FHN protein.

Total IgG (Fig 43)

To detect specific anti-FHN total IgG, ELISA plates were coated with 200ng of immunoaffinity purified FHN protein, plates were then saturated and dilutionsof the mice second bleed sera were then applied. Total IgG were detected using a biotinylated serum directed against mouse IgG.

IgG1 (Fig 44)

To detect specific anti-FHN IgG1, ELISA plates were coated with 100ng of immunoaffinity purified FHN protein, plates were then saturated and dilutionsof the mice second bleed sera were then applied. IgG1 were detected using a biotinylated serum directed against mouse IgG1.

IgG2a (Fig 45)

To detect specific anti-FHN IgG2a, ELISA plates were coated with 100ng of immunoaffinity purified FHN protein, plates were then saturated and dilutionsof the mice second bleed sera were then applied. IgG2a were detected using a biotinylated serum directed against mouse IgG2a.

The titer of each sera was determined and a mean titer for each group was calculated and is reported in table 2. These experiments show that the FHN antigen by itself or formulated with adjuvant (group 1 to 3), stimulates a specific humoral response. Indeed, no anti-FHN antibodies are generated in the untreated mice group (group 5) or in the group immunised solely with the adjuvant (group 4). The group 1 (and group 4) adjuvant was 3D-MPL and QS21 formulated with cholesterol containing liposomes as described in WO 96/33739; the group 2 adjuvant was alum.

The IgG1/IgG2a ratio indicates the Th1 or Th2 orientation of the immune response; (Table2), a protective response against both the RSV or the PiV3 should tend toward

the Th1 type, that is a low IgG1/IgG2a ratio. In this regard, the responses generated with the FHN formulated in the presence of the 3D-MPL + QS21 adjuvant appears to be the more promising one.

Table 1: Experimental procedures

Immunogenicity FHN in mice

Group	n	Vol (µl)	route	Antigen		Immuno-stimulants	buffer	preservative
				nature	dose (µg)			
1	12	2x50	IM	FHN	2	3D-MPL/ QS21	PBS mod pH 7.4	thiomersal low (1µg/ml)
2	12	2x50	IM	FHN	2	Al(OH)3	PBS mod pH 7.4	thiomersal low (1µg/ml)
3	12	2x50	IM	FHN	2	/	PBS mod pH 7.4	thiomersal low (1µg/ml)
4	12	2x50	IM	/	/	3D-MPL/ QS21	PBS mod pH 7.4	thiomersal low (1µg/ml)
5	12	/	/	untreated	/	/	/	/
6	12	2x30	INA	RSV live		/	/	/
7	12	2x30	INA	PIV-3 live		/	/	/

IM=intra-muscular

INA=intra-nasal

Antigen	cc. µg/ml	Buffer
RSV live	6.2 logPFU/ml	
PIV-3 live	6.7 logPFU/ml	
FHN	120 (2.5ml)	PBS pH 7.3

Time schedule:

Injection 1 = Day 0

Injection 2 = Day 28

First Bleed = Day 28

Second bleed = Day 42

Table 2: Serum antibody response against FHN.

The total IgG, IgG1 and IgG2a was determined for each mouse sera. A mean titer for each group was then calculated and is reported in the table.

group n°	Immunogen	Total IgG	IgG1	IgG2a	IgG1/IgG2a
1	FHN + 3D-MPL/QS21	1182000	109800	305500	0.36
2	FHN + Alum	182200	127100	4429	28.7
3	FHN	44990	22760	1941	11.73
4	adjuvant=from group 1	49	32	ND	ND
5	untreated	52	ND	ND	ND
6	Live RSV	12840	748	2718	0.27
7	Live PiV3	10860	2758	2320	1.19

ND=undetermined, the titer being to low

5

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Claims

1. A process for preparing a heterochimeric protein or an immunogenic derivative thereof comprising an immunogenic fragment of the fusion (F) protein of RSV, PIV1, PIV2, PIV3, MV or MuV and an immunogenic fragment of the attachment (G, HN or H) protein of RSV, PIV1, PIV2, PIV3, MV or MuV which process comprises expressing recombinant DNA encoding the heterochimeric protein or immunogenic derivative thereof in CHO cells and recovering the protein.

2. A process according to claim 1 wherein at least one non-preferred or less preferred codon in a natural gene or DNA encoding the said heterochimeric protein or immunogenic fragment thereof has been replaced by a preferred codon encoding the same amino acid.

3. A heterochimeric protein or an immunogenic derivative thereof comprising an immunogenic fragment of the fusion (F) protein of RSV, PIV1, PIV2, PIV3, MV or MuV and an immunogenic fragment of the attachment (G, HN or H) protein of RSV, PIV1, PIV2, PIV3, MV or MuV, with the proviso that where one of the immunogenic fragments is derived from RSV F, RSV G or PIV3 F, PIV3 HN, the other of the immunogenic fragments is derived from MuV F, MuV HN, MV F, MV H, PIV1 F,PIV1 HN, PIV2 F or PIV2 HN.

4. A process for preparing a heterochimeric protein or immunogenic derivative thereof as claimed in claim 3 which process comprises expressing recombinant DNA encoding the heterochimeric protein or immunogenic derivative thereof in either one of; CHO cells or insect cells and recovering the protein.

5. A protein according to claim 3 wherein the immunogenic fragment of the F protein is lacking the membrane anchor domain at its C-terminal end.

6. A protein according to claims 3 or 5 wherein the immunogenic fragment of the G, HN or H protein is lacking the signal/anchor domain at its N-terminal end.

7. A protein according to any one of claims 3, 5 or 6 which is linked *via* an amino acid in the C-terminal part of the immunogenic fragment of the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV to an amino acid in the N-terminal part of the immunogenic fragment of the G protein of RSV or the HN protein of PIV1, PIV2, PIV3, MuV or the H protein of MV.

8. A protein according to any one of claims 3, 5, 6 or 7 which commences at its N-terminal end with a signal sequence from the F protein of RSV, PIV1, PIV2, PIV3, MV or MuV.

9. A protein according to any one of claims 3,5,6 or 7 which commences at its N-terminal end with a signal sequence from TPA.

10. A protein according to any one of claims 3 or 5 to 8 which comprises a ubiquitin leader sequence.

11. A protein according to any one of claims 3 or 5 to 9 which comprises a polyhistidine tail.

12. A protein according to claim 10 or 11 which comprises a cleavage site for cleaving off the ubiquitin leader sequence and/or the polyhistidine tail.

13. A heterochimeric protein according to any one of claims 3 or 5 to 11 which is selected from the group consisting of:

$Fs^{+}a^{-}RSVxHNs^{-}a^{-}MuV$;

$Fs^{+}a^{-}$ PIV3 x $HNs^{-}a^{-}$ MuV;

$Fs^{+}a^{-}$ MuV x $Gs^{-}a^{-}RSV$; or

$Fs^{+}a^{-}$ MuV x $HNs^{-}a^{-}$PIV3, or

an immunogenic derivative thereof.

14. A heterochimeric protein according to any one of claims 3 or 5 to 11 which is selected from the group consisting of:

Fs^+a^- MuV x Hs^-a^-MV; or

Fs^+a^-RSVx HNs^-a^-PIV1, or

Fs^+a^-RSVx HNs^-a^-PIV2, or

an immunogenic derivative thereof.

15. A heterochimeric protein which is:

Fs^+a^- (1-526) RSV x HNs^-a^- (70-572) PIV3,

Fs^+a^- (1-492) PIV3 x Gs^-a^- (69-298) RSV,

Fs^+a^- (1-526) RSV x HNs^-a^- (70-572) PIV3 bis,

Fs^+a^- (1-526) RSV x HNs^-a^- (70-572) PIV3 ent his, or

sTPA (1-21) UB (1-74) ent Fs^-a^- (24-526) x HN s^-a^-(70-572) PIV3, or

an immunogenic derivative thereof.

16. Recombinant DNA encoding a heterochimeric protein or an immunogenic derivative thereof according to any one of claims 3 or 5 to 15.

17. Recombinant DNA according to claim 16 in which at least one non-preferred or less preferred codon in the DNA has been replaced by a preferred codon encoding the same amino acid.

18. DNA which hybridises under conditions of high stringency with the DNA of claim 16 or 17.

19. An expression vector comprising recombinant DNA according to claims 16 to 18.

20. A host transformed with DNA according to any one of claims 16 to 18 or with a vector according to claim 19.

21. A host according to claim 20 which is a CHO cell.

22. A host according to claim 21 which is an insect cell.

23. A vaccine composition comprising a protein according to any one of claims 3 or 5 to 13 or an immunogenic derivative thereof in admixture with a pharmaceutically acceptable carrier.

24. A vaccine composition according to claim 23 further comprising 3D Monophosphoryl lipid A and/or QS-21.

25. A vaccine composition according to claims 23 or 24 wherein the carrier is an oil-in-water emulsion.

26. A heterochimeric protein or an immunogenic derivative thereof according to any one of claims 3 or 5 to 15 for use in medicine.

27. A process for the production of a heterochimeric protein according to any one of claims 3 or 5 to 15 which process comprises expressing recombinant DNA encoding said protein or immunogenic fragment thereof in a host cell and recovering the protein.

28. A method of treating a human or animal susceptible to paramyxoviridae viral infections comprising administering an effective amount of a vaccine according to any one of claims 23 to 25.

29. Use of a protein or an immunogenic derivative thereof according to any one of claims 3 or 5 to 15 in the manufacture of a medicament for use in the treatment of respiratory disorders.

Fig. 1

pNIV2819

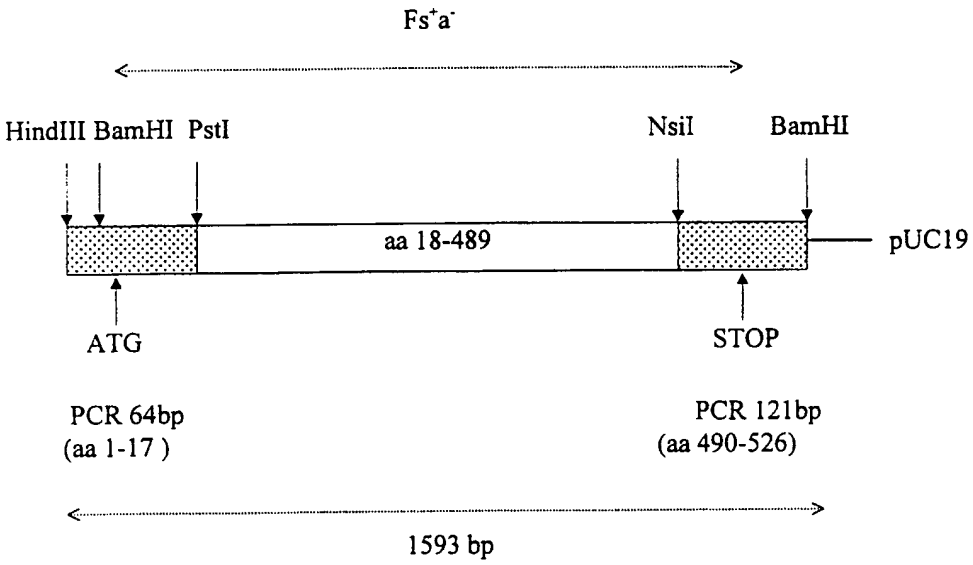


Fig. 2

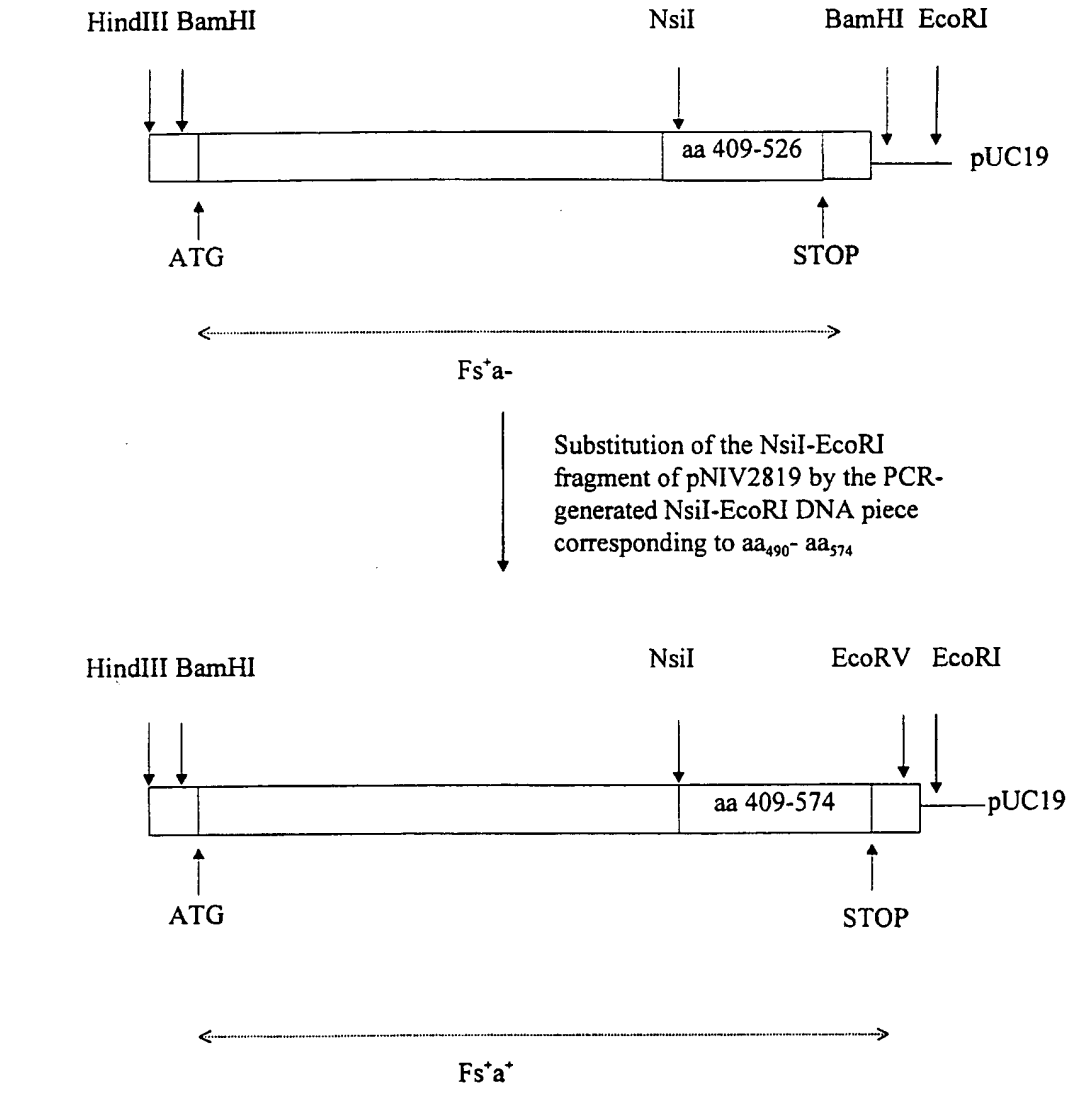

pNIV2820
HindIII BamHI
NsiI
BamHI EcoRI
aa 409-526
pUC19
ATG
STOP
Fs+a-
Substitution of the NsiI-EcoRI fragment of pNIV2819 by the PCR-generated NsiI-EcoRI DNA piece corresponding to aa490- aa574
HindIII BamHI
NsiI
EcoRV EcoRI
aa 409-574
pUC19
ATG
STOP
Fs+a+

Fig. 3

pNIV2841

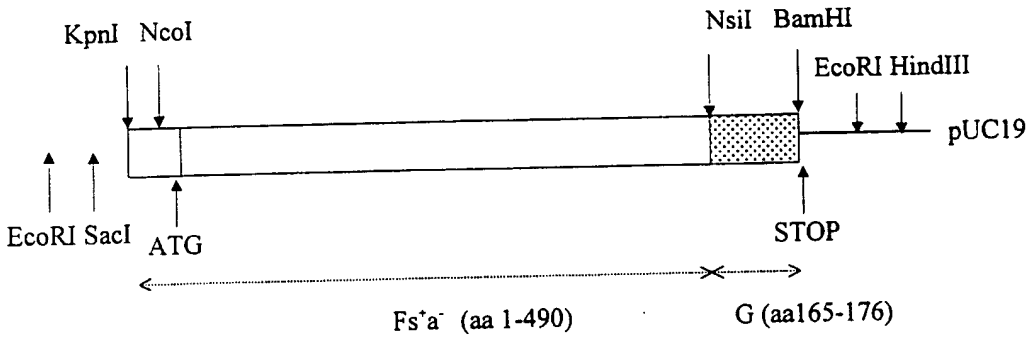


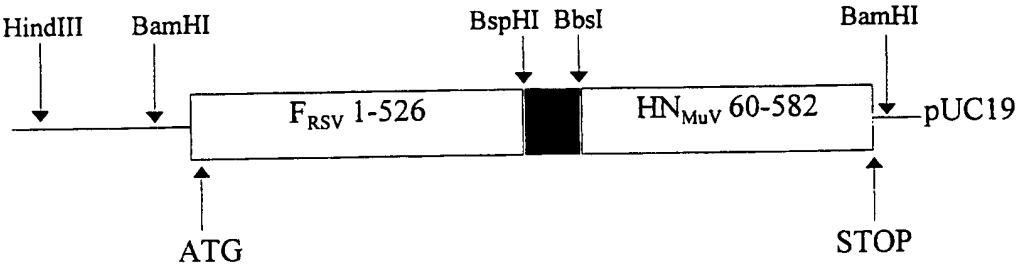
Fig. 4

A) Synthetic adaptators

```
5' C ATG AAT GAT CAA GGC TTG AGC AA 3'
       TTA CTA GTT CCG AAC TCG TTA GTC   [SEQ ID NO: 1]
```

BspHI BbsI

B) pNIV4102



C) pNIV4104

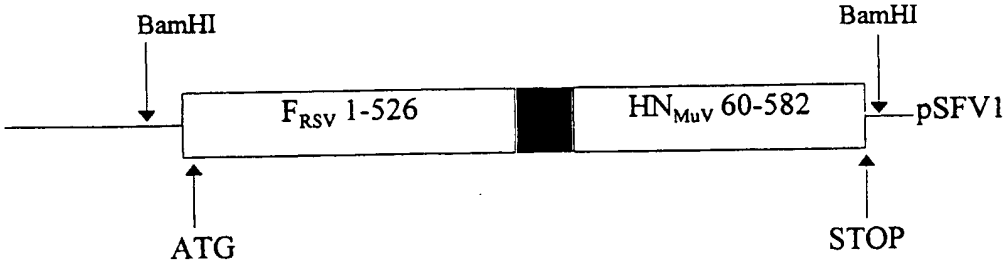


Fig. 5

A) Synthetic adaptators

```
5' C ATG AAC AAT GAG TTT ATG GAA GTT ACA GAA AAG ATC CAA
       TTG TTA CTC AAA TAC CTT CAA TGT CTT TTC TAG GTT
  BspHI

ATG GCA TCG GAT ATT AT 3'
TAC CGT AGC CTA TAA TATA     [SEQ ID NO: 2]
                  AseI
```

B) pNIV4109

C) pNIV4110

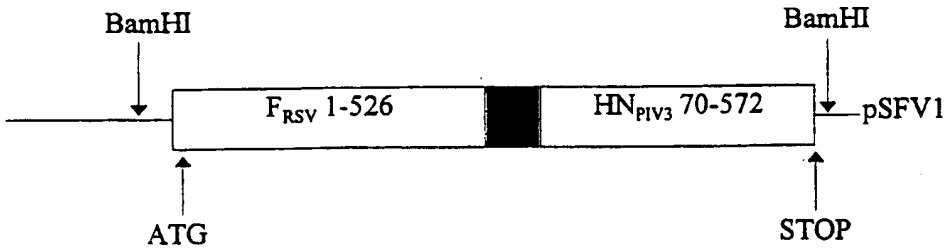


Fig. 6

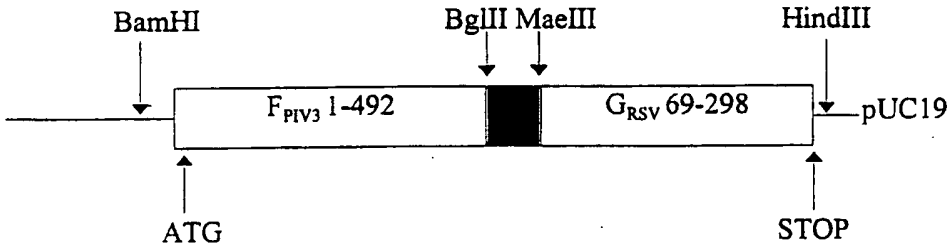
A) Synthetic adaptators

```
5' GAT CTA GAA GAG TCA AAA GAA TGG ATA AGA AGG TCA AAT CAA
       AT CTT CTC AGT TTT CTT ACC TAT TCT TCC AGT TTA GTT
  BglII

AAA CTA GAT TCC ATT GGA AAT TGG CAT CAA TCT AGC ACC 3'
TTT GAT CTA TGG TAA CCT TTA ACC GTA GTT AGT TCG TGG CAGT G
                                                  MaeIII
```

[SEQ ID NO: 3]

B) pNIV4103



C) pNIV4106

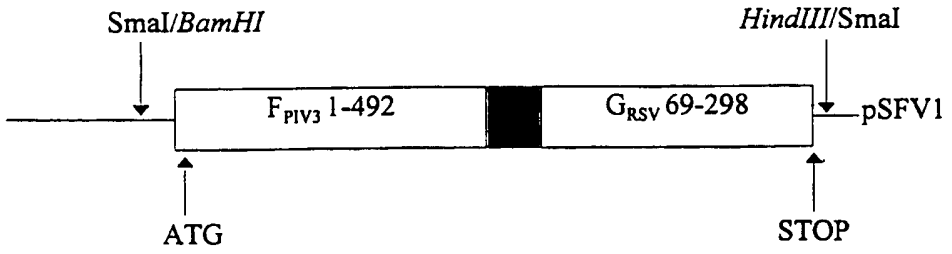


Fig. 7

A) Synthetic adaptators

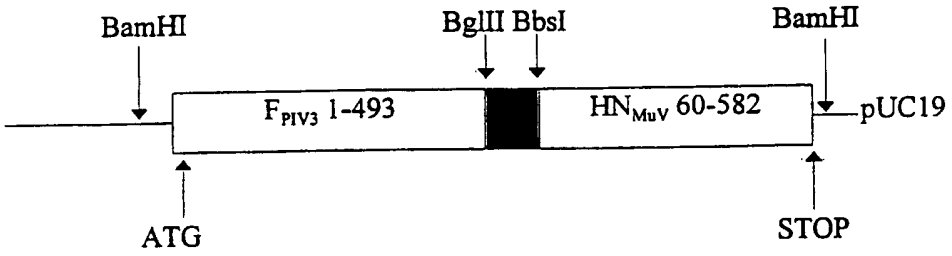
```
5'G ATC TAG AAG AGT CAA AAG AAT GGA TAA GAA GGT CAA ATC
       ATC TTC TCA GTT TTC TTA CCT ATT CTT CCA GTT TAG
```

BglII

```
AAA AAC TAG ATT CCA TTG GAA ATT GGC ATC AAT CTA GCA CCA
TTT TTG ATC TAA GGT AAC CTT TAA CCG TAG TTA GAT CGT GGT


CAA ATG ATC AAG GCT TGA GCA A 3'
GTT TAC TAG TTC CGA ACT CGT TAGTC      [SEQ ID NO: 4]
                          BbsI
```

B) pNIV4117



C) pNIV4118

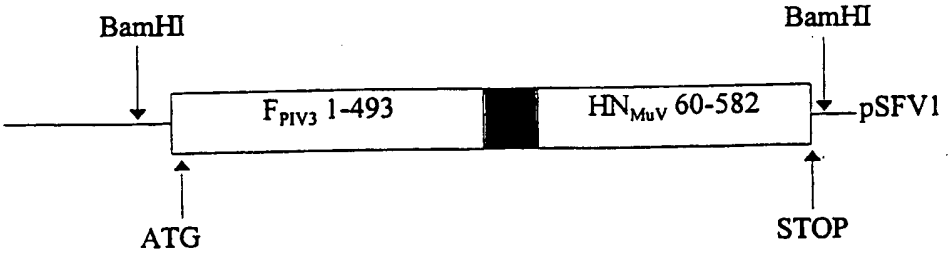


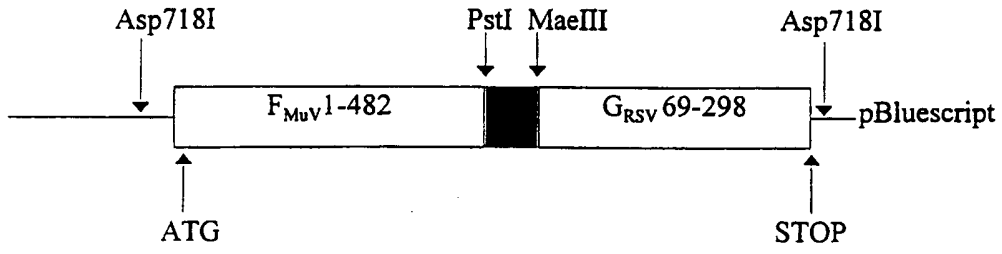
Fig. 8

A) Synthetic adaptators

```
5'      GAA TGC CGT TAA ATA CAT CAA GAG AGT AAC CAT CAA
  A CGT CTT ACG GCA ATT TAT GTA GTT CTC TCA TTG GTA GTT
 PstI

CTC CAT CGG TCT CAG TAA GTT CTA AA  3'
GAG GTA GCC AGA GTC ATT CAA GAT TTC AGT        [SEQ ID NO: 5
                                        MaeIII
```

B) pNIV4113



C) pNIV4114

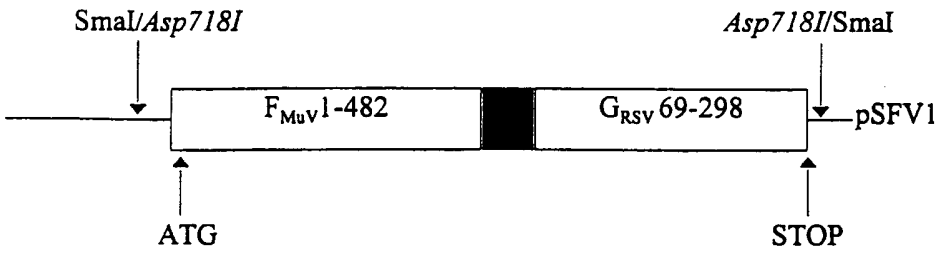
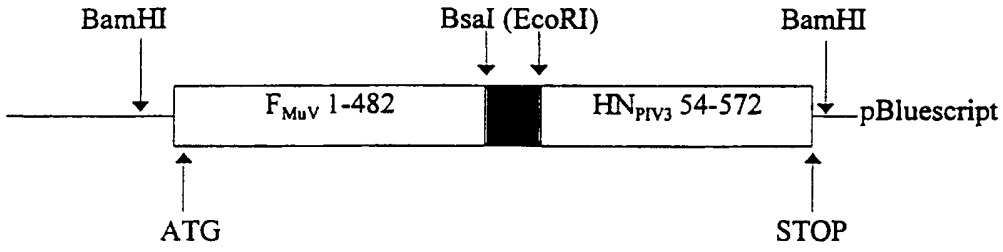


Fig. 9

A) Synthetic adaptators

5' GTAAGTTCTAAA 3'
CAAGATTTTTAA [SEQ ID NO: 6]
BsaI EcoRI

B) pNIV4115



C) pNIV4116

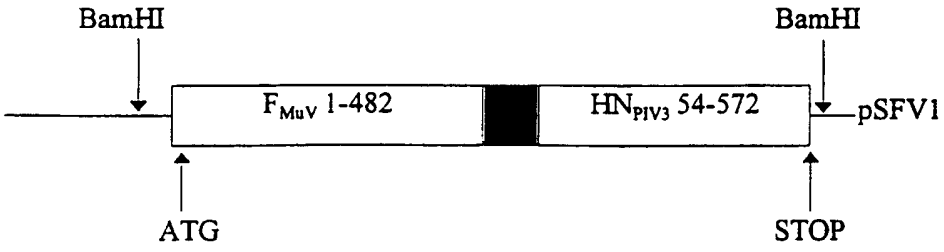
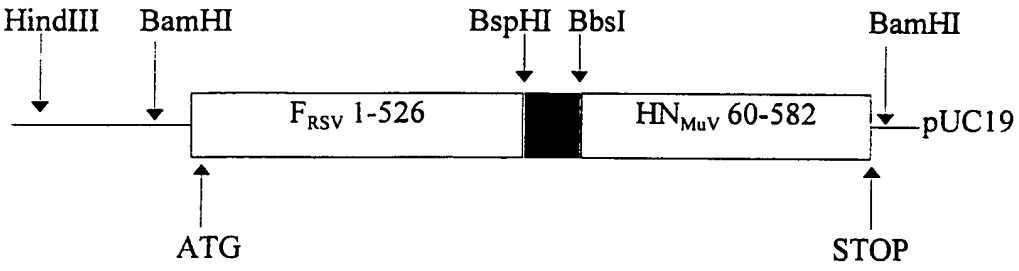


Fig. 10

A) pNIV4102



C) pEE14 $Fs^{+}a^{-}$ RSV x HN $s^{-}a^{-}$ MuV

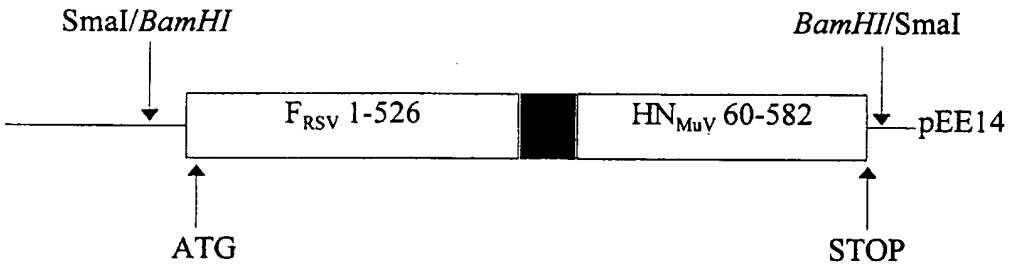
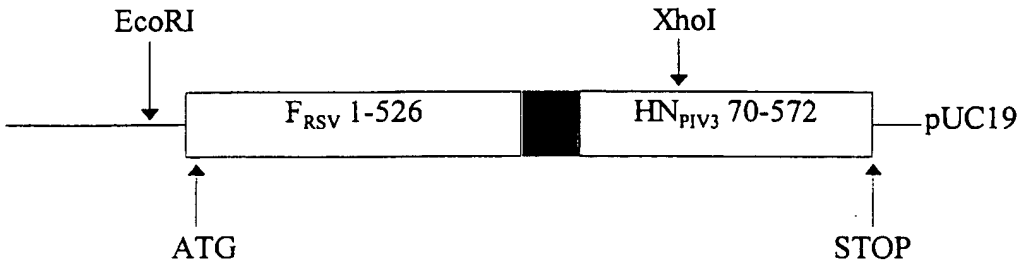
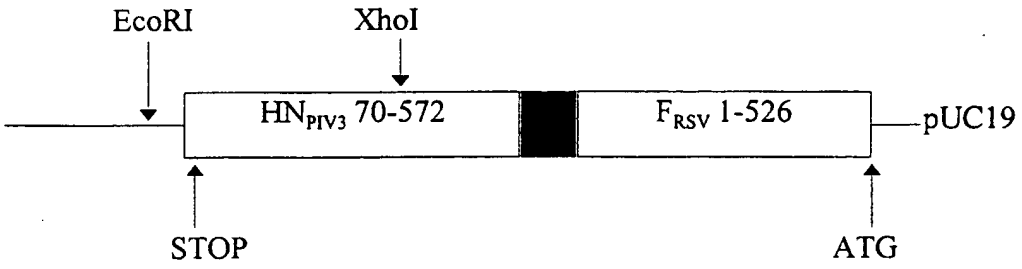


Fig. 11

A) pNIV4105



B) pNIV4109



C) pEE14 $Fs^{+}a^{-}$ RSV x HN $s^{-}a^{-}$ PIV3

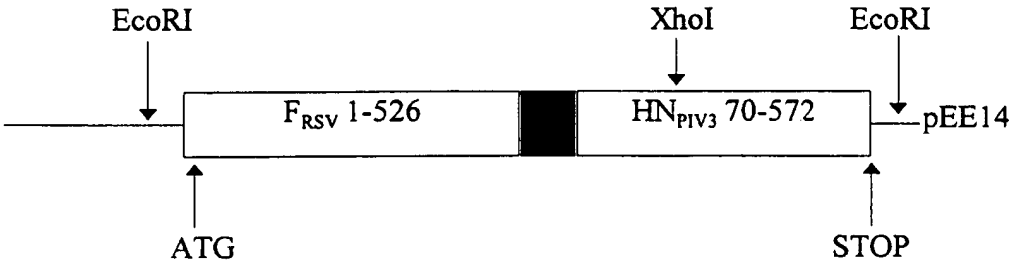
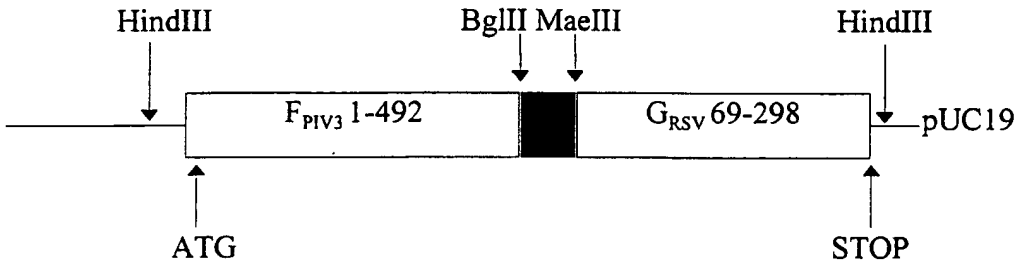


Fig. 12

A) pNIV4103



B) pEE14 $Fs^{+}a^{-}$ PIV3 x G $s^{-}a^{-}$ RSV

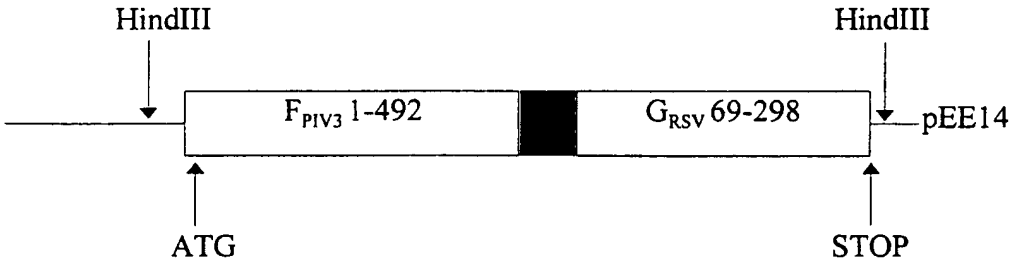
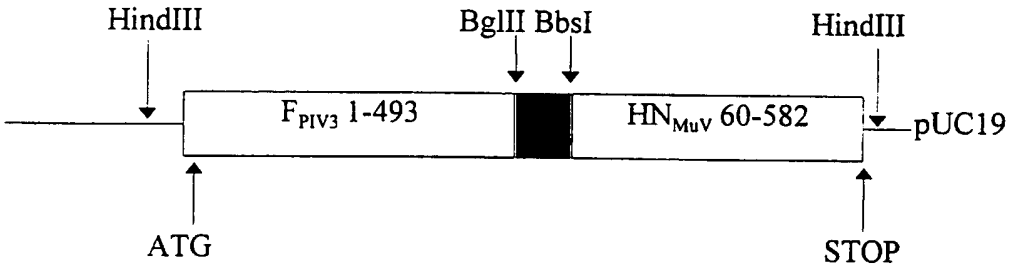


Fig. 13

A) pNIV4117



B) pEE14 $Fs^{+}a^{-}$ PIV3 x HN $s^{-}a^{-}$ MuV

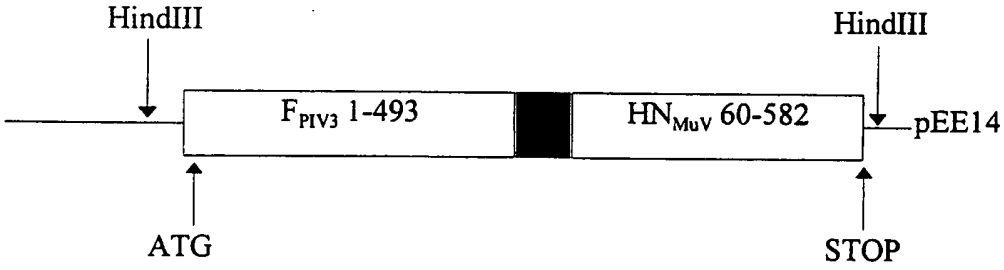
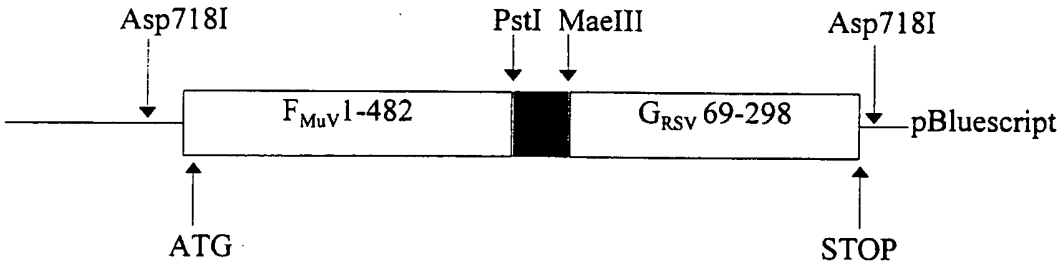


Fig. 14

A) pNIV4113



B) pEE14 $Fs^{+}a^{-}$ MuV x G $s^{-}a^{-}$ RSV

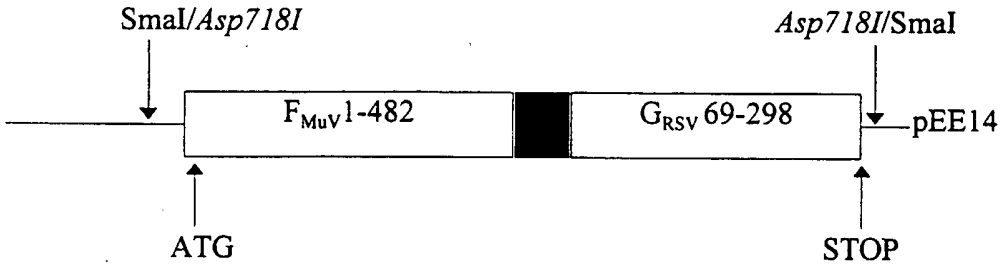
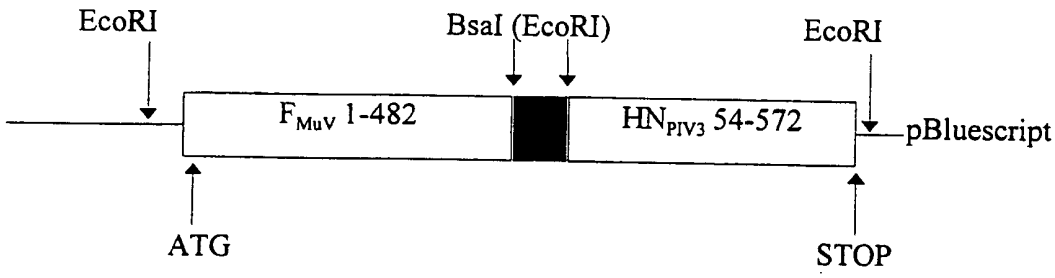


Fig. 15

A) pNIV4115



B) pEE14 $Fs^{+}a^{-}$ MuV x $HNs^{-}a^{-}$ PIV3

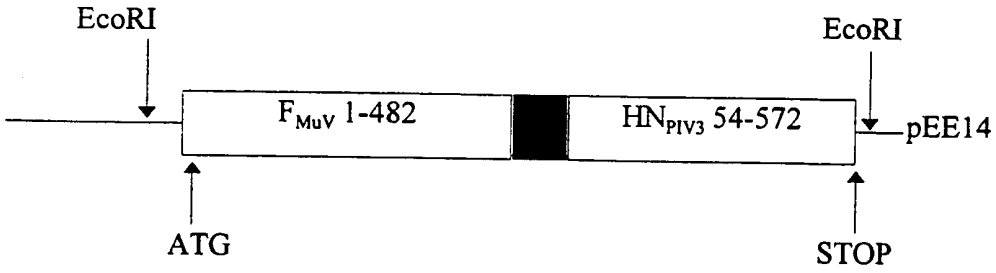
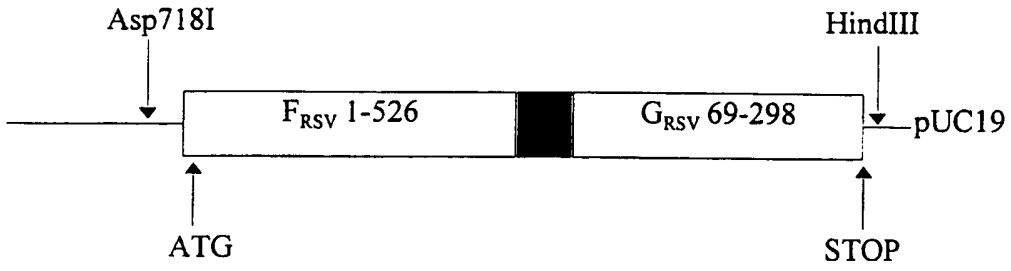


Fig. 16

A) pNIV2857



B) pNIV2870

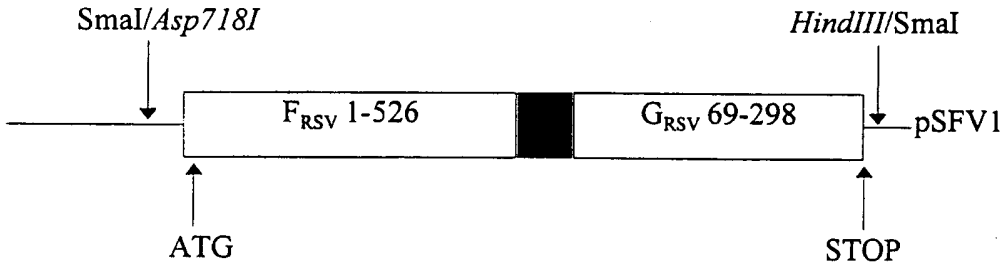
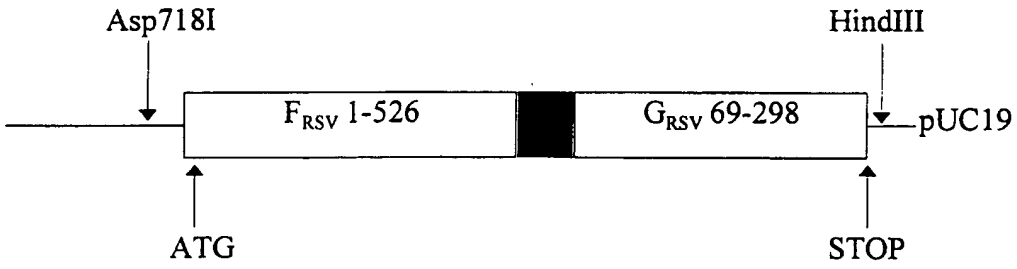


Fig. 17

A) pNIV2857



B) pEE14 $Fs^{+}a^{-}$ RSV x G $s^{-}a^{-}$ RSV

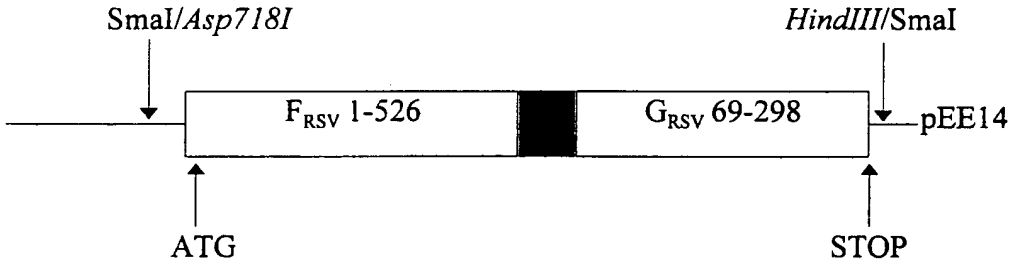


Fig. 18

A) Synthetic adaptators

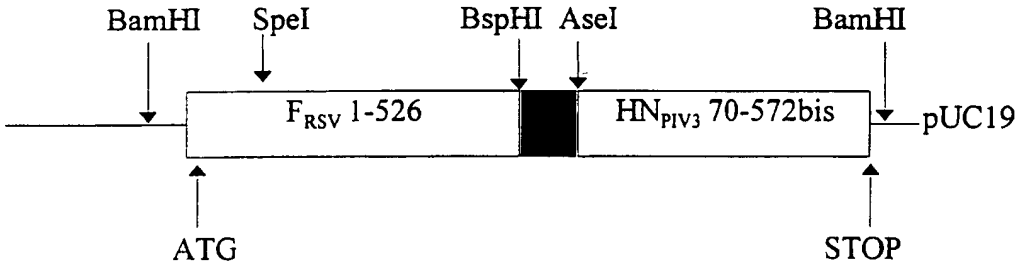
```
5' C ATG AAC AAT GAG TTT ATG GAA GTT ACA GAA AAG ATC CAA
       TTG TTA CTC AAA TAC CTT CAA TGT CTT TTC TAG GTT
```

BspHI

```
ATG GCA TCG GAT ATT AT 3'
TAC CGT AGC CTA TAA TAT A      [SEQ ID NO: 7]
```

AseI

B) pNIV4120



C) pEE14 F s^+ a^- RSV xHN s^-a^- PiV3bis

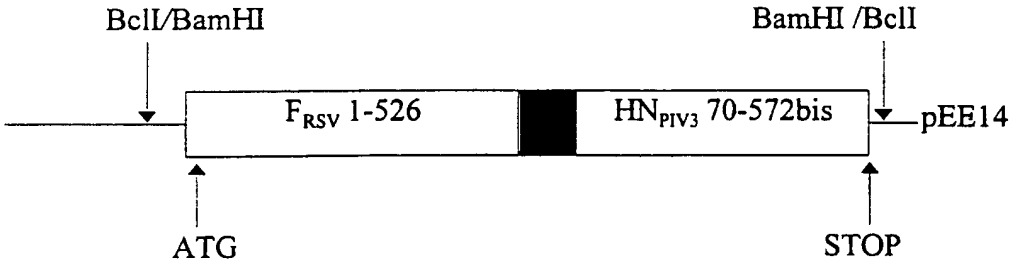


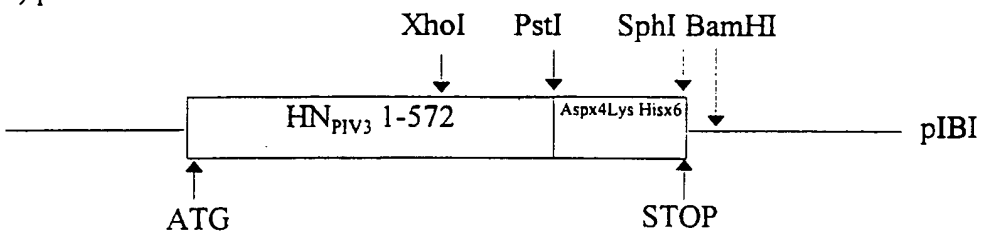
Fig. 19

A) Synthetic adaptators

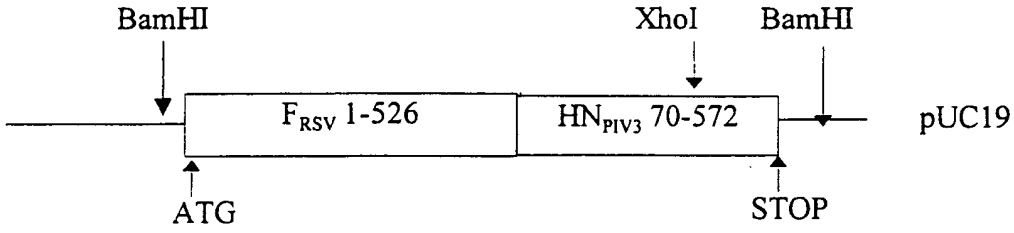
PstI 5'GT GAC GAT GAC GAT AAG CAT CAT CAT CAT CAT CAT TAG
ACGTC ACA CTG CTA CTG CTA TTC GTA GTA GTA GTA GTA GTA ATC

GGATCCGCATG 3'
CCTAGGC SphI [SEQ ID NO: 8]

B) pNIV3340



C) pNIV4120



C) pEE14 F s$^+$ a$^-$ RSV xHN s$^-$a$^-$ PiV3 enthis

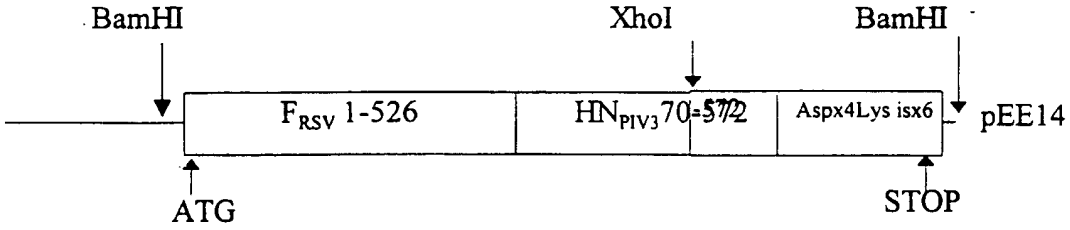
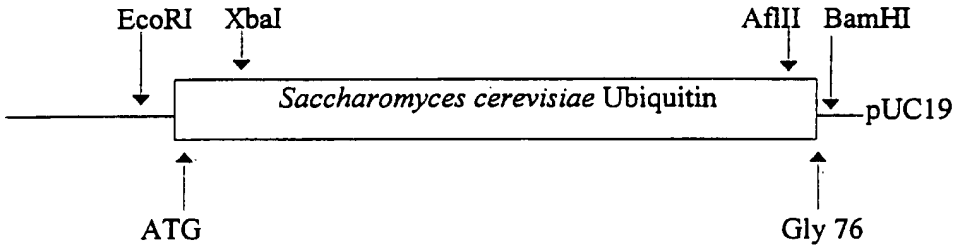


Fig. 20

A) pNIV3475



B) Synthetic adaptators

5' CT AGC ATG CAG ATC TTC GTC AAG ACG TTA ACC GGT AAA ACC
NheI G TAC GTC TAG AAG CAG TTC TGC AAT TGG CCA TTT TGG

ATA ACC 3' XbaI
TAT TGG ATCT [SEQ ID NO: 9]

C) pNIV4122

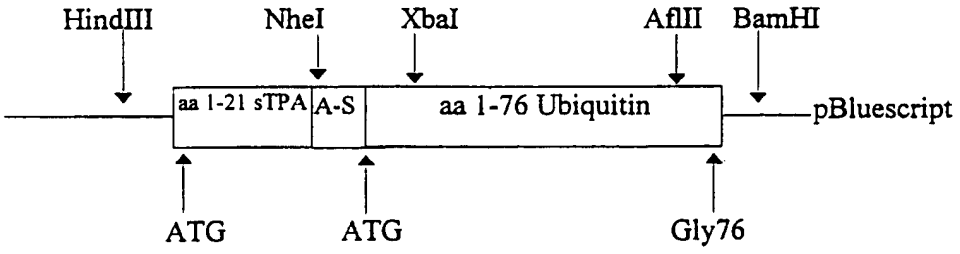
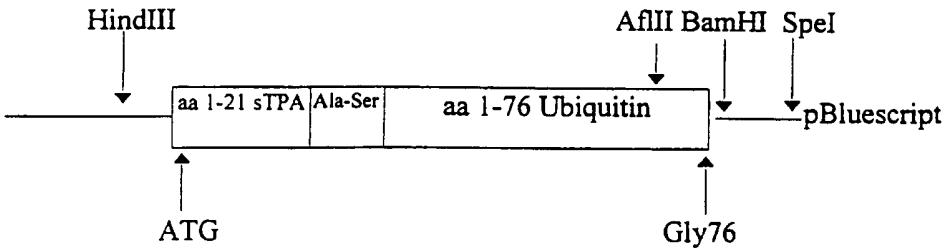


Fig. 21

A) pNIV4122



B) Synthetic adaptators

5' TTA AGA CTA AGA GAC GAT GAC GAT AAG TCC AGT CAA AAC
AflII CT GAT TCT CTG CTA CTG CTA TTC AGG TCA GTT TTG

ATC ACT GAA GAA TTT TAT CAA TCA ACA TGC AGT GCA GTC AGC
TAG TGA CTT CTT AAA ATA GTT AGT TGT ACG TCA CGT CAG TCG

AAA GGC TAT CTT AGT GCT CTA AGA ACT GGT TGG TAT A 3' SpeI
TTT CCG ATA GTT TCT CGA GAT TCT TGA CCA ACC ATA TGA TC

[SEQ ID NO: 10]

C) pNIV4123

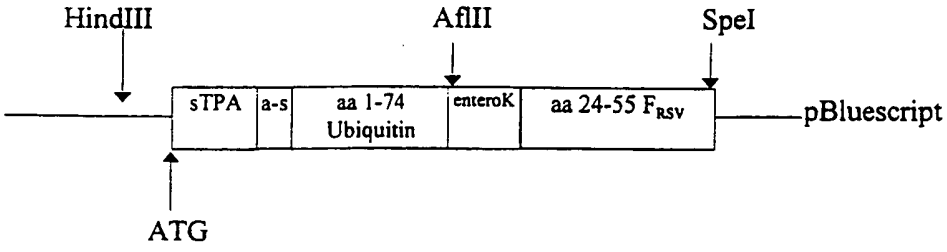
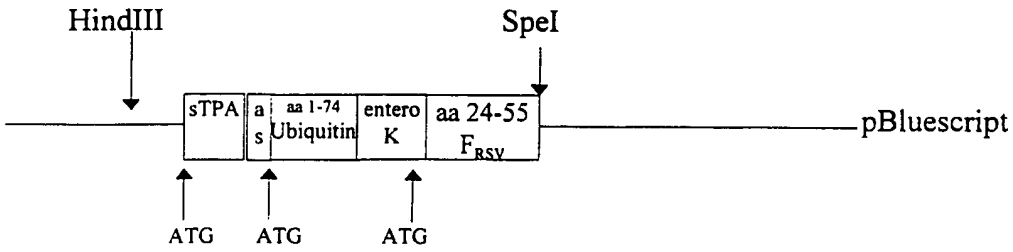
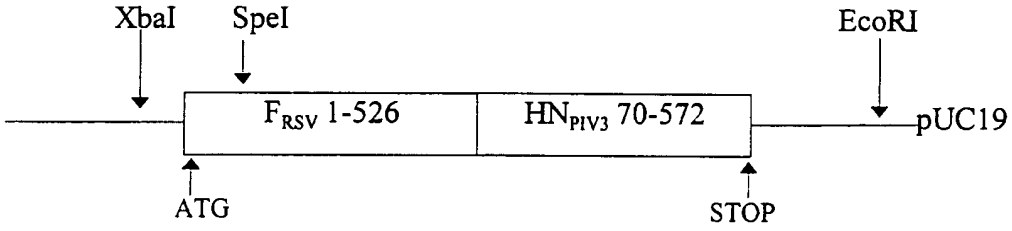


Fig. 22

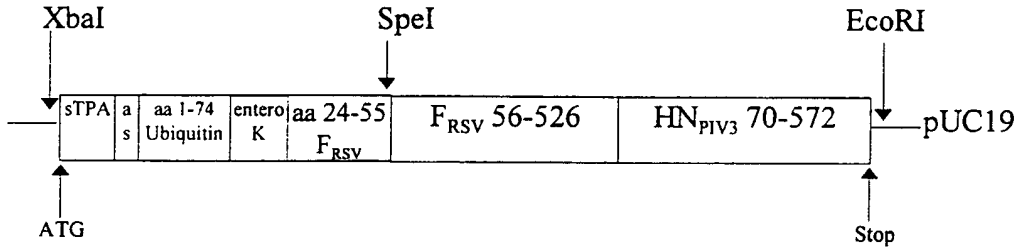
A) pNIV4123



B) pNIV4120



C) pNIV4124



D) pEE14 sTPA UBI EN $Fs^{-}a^{-}$ RSV x HN $s^{-}a^{-}$ PiV3

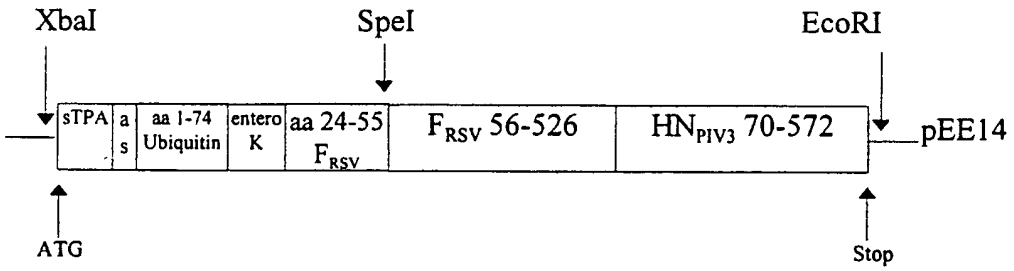
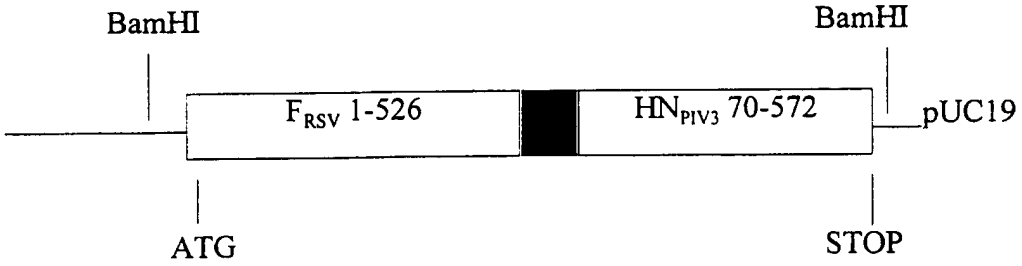


Fig. 23

A) pNIV4120



B) pNIV4132

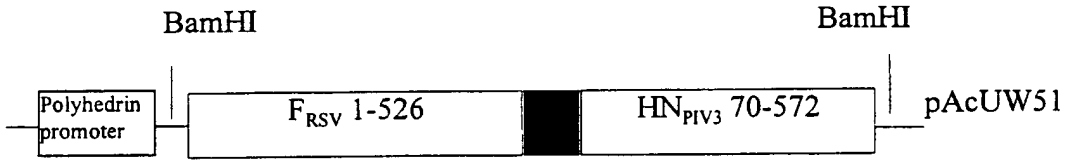
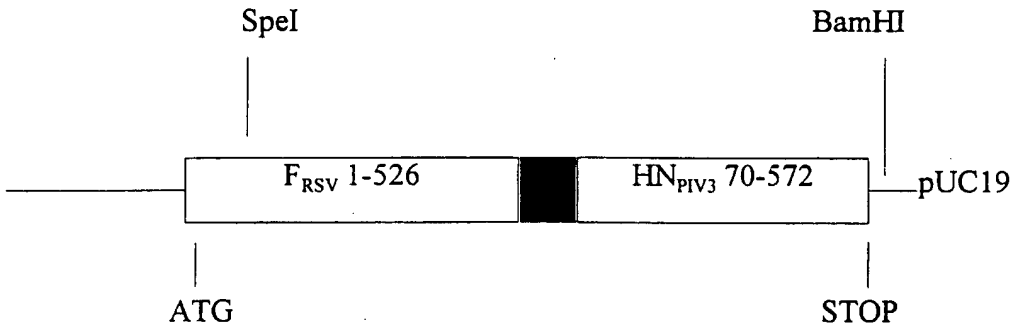


Fig. 24

A) pNIV4120



B) Synthetic adaptators

```
5'GAT CAA AAC ATC ACT GAA GAA TTT TAT CAA TCA ACA TGC
 BamHI TT TTG TAG TGA CTT CTT AAA ATA GTT AGT TGT ACG

AGT GCA GTC AGC AAA GGC TAT CTT AGT GCT CTA AGA ACT
TCA CGT CAG TCG TTT CCG ATA GAA TCA CGA GAT TCT TGA

GGT TGG TAT A 3'SpeI
CCA ACC ATA TGA TC  [SEQ ID NO: 11]
```

C) pNIV4136

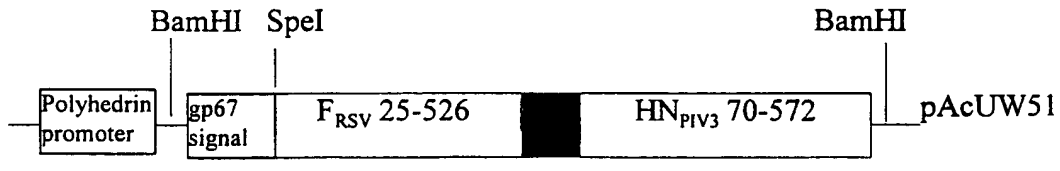


Fig 25: SDS-PAGE (reduced conditions) of the $F_{RSV}HN_{PIV3}$ protein purified by immunoaffinity from the spent culture medium of the recombinant baculovirus 3546.

kDa: molecular weight marker

A: Coomassie blue staining
B: Western blot revealed by a goat polyclonal anti-RSV serum 20 RG45

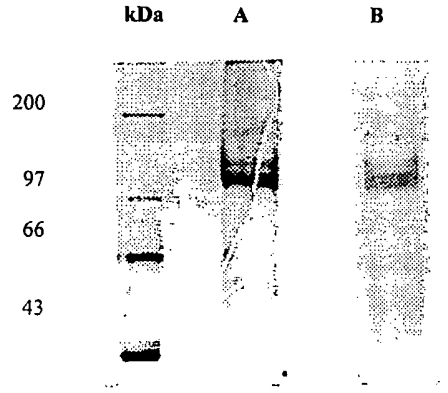


Fig . 26: Codon usage of $F_{RSV}HN_{PiV3}$ and highly expressed human genes (hum high exp) showing frequencies (x100) of the individual codons for each of the degenerately encoded amino acids, and the most prevalent codon in bold.

		hum high exp	$F_{RSV}HN_{PiV3}$
Ala	GCG	17	3
	A	13	**51**
	T	17	35
	C	**53**	11
Arg	AGG	18	28
	A	10	**56**
	CGG	21	0
	A	6	10
	T	7	5
	C	**37**	0
Asn	AAT	22	**62**
	C	**78**	38
Asp	GAT	25	**66**
	C	**75**	34
Cys	TGT	32	**62**
	C	**68**	38
Gln	CAG	**88**	32
	C	12	**68**
Glu	GAG	75	22
	A	25	**77**
Gly	GGG	24	22
	A	14	**37**
	T	12	22
	C	**50**	19
His	CAT	21	**86**
	C	**79**	14
Ile	ATA	5	**49**
	T	18	30
	C	**77**	20
Leu	TTG	6	10
	A	2	**39**
	CUG	**58**	6
	A	3	23
	T	5	11
	C	26	12
Lys	AAG	**82**	34
	A	18	**66**
Phe	TTT	20	**63**
	C	**80**	37
Pro	CCG	17	12
	A	16	**51**
	T	19	26
	C	**48**	12
Ser	AGT	10	**21**
	C	**34**	16
	TCG	9	7
	A	5	**27**
	T	13	17
	C	28	13
Thr	ACG	15	6
	A	14	**56**
	T	14	26
	C	**57**	12
Tyr	TAT	26	**79**
	C	**74**	21
Val	GTG	**64**	19
	A	5	**41**
	T	7	23
	C	25	17

Fig. 27: Schematic diagram of the PCR synthesis of each fragment showing unique restriction sites along the sequence (black dots) and restriction sites (A and B) that allow retrieval of the full size fragment from the cloning vector.

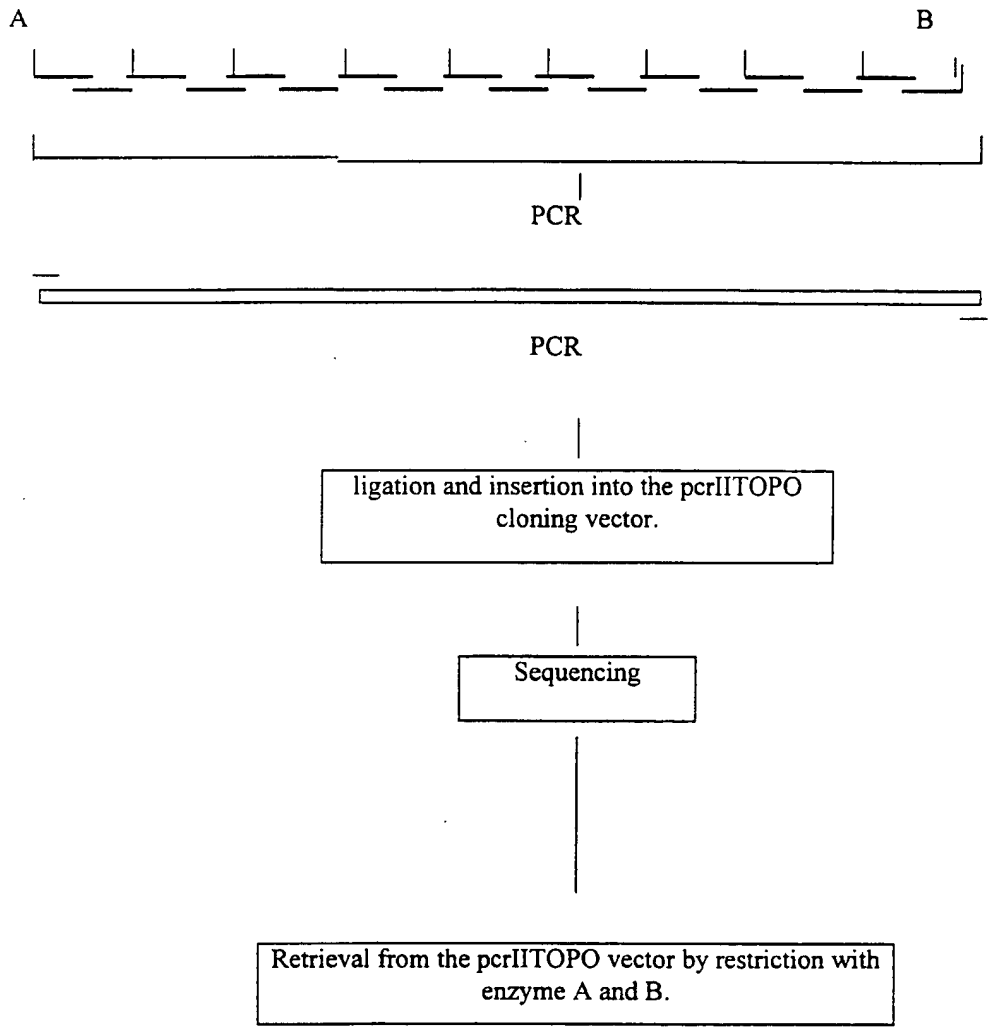


Fig. 28: Sequence of the 18 oligonucleotides from which PCR fragment A was generated.

1) olfhum1.seq, bases 1-90 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'cccTCTAGAGGATCCACCATGGAGCTGCTGATtttaAAGACCAACGCCATCACCGCCATCCTG
GCCGCGGTGACCCTCTGCTTCGCGTCC

2) olfhum2.seq, bases 75-165 of $F_{RSV}HN_{PiV3}$, inverse complementary to mARN
5'CCTCAGCGCGCTCAGGTAGCCCTTGCTGACAGCagaGCAGGTGGACTGGTAGAACTCCTCGGTG
ATGTTCTGGCTGGACGCGAAGCAGAGG

3) olfhum3.seq, bases 150-240 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'CCTGAGCGCGCTGAGGACGGGGTGGTACACtAGtGTGATCACCATCGAGCTGAGCAACATCAAG
GAGAACAAGTGCAACGGCACCGACGCC

4) olfhum4.seq, bases 225-310 of $F_{RSV}HN_{PiV3}$, inverse complementary to mARN
5'GCATCAGCAGCTGCAGCTCGGTCACGGCGCTCTTGTACTTGTCCAGCTCCTGCTTGATCAGCTT
CACCTTGGCGTCGGTGCCGTTG

5) olfhum5.seq, bases 295-397 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'CTGCAGCTGCTGATGCAGAGCACCCCCGCCACCAACAACagaGCCAGGCGCGAGCTGCCCAGGT
TCATGAACTACACCCTCAACAACACCAAGAACACCAACG

6) olfhum6.seq, bases 378-496 of $F_{RSV}HN_{PiV3}$, inverse complementary to mRNA
GGTGCAGGACCTTGGACACCGCGATGCCGCTGGCGATGGCGGAGCCCACGCCCAGCAGGAAGCCCA
GGAAgCGcctCTTgCgCTTCTTGCTCAGGGTCACGTTGGTGTTCTTGGTGTTG

7) olfhum7.seq, bases 480-561 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'GTCCAAGGTCCTGCACCTGGAGGGGGAGGTGAACAAGATCAAGAGCGCCCTGCTCTCCACCAAC
AAGGCGGTGGTCAGCCTG

8) olfhum8.seq, bases 543-633 of $F_{RSV}HN_{PiV3}$, inverse complementary to mARN
5'GGGGAGCAatTGCTTGTCGATGTAGTTCTTGAGGTCCAGCACCTTGCTGGTCAGCACGCTCACG
CCGTTGGACAGGCTGACCACCGCCTTG

9) olfhum9.seq, bases 609-676 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'CTACATCGACAAGCAatTGCTCCCCATCGTGAACAAGCAGtcCTGCAGCATCTCTAACATTGAG
ACCG

10) olfhum10.seq, bases 653-732 of $F_{RSV}HN_{PiV3}$, inverse complementary to mARN
5'GCTGAACTCCCTGGTGATCTCCAGCAGCCTGTTGTTCTTCTGCTGGAACTCGATCACGGTCTCA
ATGTTAGAGATGCTGC

11) olfhum11.seq, bases 714-787 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'GATCACCAGGGAGTTCAGCGTGAACGCgGGcGTcACCACCCCGGTGAGCACCTACATGCTGACC
AACAGCGAGC

12) olfhum12.seq, bases 768-846 of $F_{RSV}HN_{Piv3}$, inverse complementary to mARN
5'GTTGGACATaAGCTTCTTCTGGTCGTTGGTGATGGGCATGTCGTTGATCAGGGACAGCAGCTCG
CTGTTGGTCAGCATG

13) olfhum13.seq, bases 825-916 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'CCAGAAGAAGCTtATGTCCAACAACGTGCAGATCGTGCGCCAGCAGAGCTACagCATCATGagC
ATCATCAAGGAGGAGGTGCTGGCCTACG

14) olfhum14.seq, bases 900-990 of $F_{RSV}HN_{Piv3}$, inverse complementary to mARN
5'GGTGGTGCACAGGGGGGAGGTGTGCAGCTTCCAGCAGGGGGTGTCGATCACGCCGTACAGGGGC
AGCTGCACCACGTAGGCCAGCACCTCC

15) olfhum15.seq, bases 975-1065 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'CCCCCTGTGCACCACCAACACCAAGGAGGGCTCCAACATCTGCCTGACCCGCACCGACCGGGGC
TGGTACTGCGACAACGCCGGCTCCGTG

16) olfhum16.seq, bases 1048-1133 of $F_{RSV}HN_{Piv3}$, inverse complementary to mARN
5'CTGTTCATGGTGTCGCAGAACACGCGGTTGGACTGCACCTTGCAGGTCTCCGCCAGGGGGAAGA
AGGACACGGAGCCGGCGTTGTC

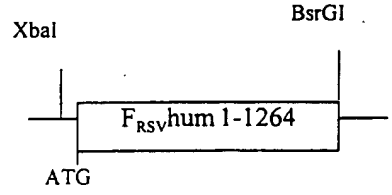
17) olfhum17.seq, bases 1116-1210 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'CTGCGACACCATGAACAGCCTGACCCTGCCCAGCGAGGTGAACCTCTGCAACATCGACATCTTC
AACCCCAAGTACGACTGCAAGATtATGacctcc

18) olfhum18.seq, bases 1195-1295 of $F_{RSV}HN_{Piv3}$, inverse complementary to mARN
gggaattctgtacacttggtcttgccgtagcaggacacgatggcgcccagggaggtgatcacggag
ctgctcacgtcggtcttggaggtCATaATCTTGCAG

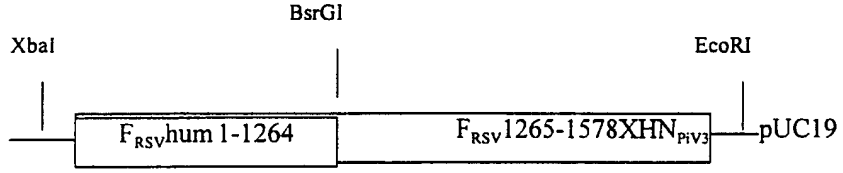
[SEQUENCES ABOVE ARE SEQ ID NOs: 12 to 29, respectively]

Fig. 29: Construction of pEE14 F_{RSV}humHN$_{PiV3}$.

a) PCR fragment A



b) pNIV4120 +PCR fragment A



c) pEE14 F_{RSV}hum HN$_{PiV3}$

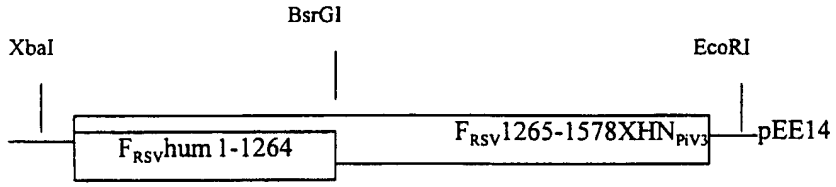


Fig. 30: Sequence of the 10 oligonucleotides from which PCR fragment B was generated.

1) olfhnhum19.seq, bases 1269-1353 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'cggcaagaccaagtgtacagcctccaacaagaaccgcggcatcatcaagaccttctccaacgg
ctgcgactacgtgtccaacaag 3'

2)olfhnhum20.seq, bases 1336-1428 of $F_{RSV}HN_{Piv3}$, inverse
complementary of mARN
5'cttcacgtacaggctcttgccctcctgcttgttcacgtagtacagggtgttgcccacggacac
ggtgtccacgcccttgttggacacgtagtc 3'

3)olfhnhum21.seq, bases 1413-1497 of $F_{RSV}HN_{Piv3}$, homologous to mARN
5'gagcctgtacgtgaagggcgagcccatcatcaacttctacgacccgctggtgttcccctccga
cgagttcgacgcctccatctccc 3'

4)olfhnhum22.seq, bases1483-1599 of $F_{RSV}HN_{Piv3}$, inverse
complementary of mARN
5'gttcatgatgttggtggtggacttgccggcgttcacgttgtgcagcagctcgtcggacttgcg
gatgaaggccaggctctggttgatcttctcgttcacctgggagatggaggcgtc 3'

5)olfhnhum23.seq, bases 1581-1691 of $F_{RSV}HN_{Piv3}$, homologous to mARN
5'caccaccaacatcatgaacaacgagttcatggaggtgaccgagaagatccagatggcctccga
caacatcaacgacctgatccagtccggcgtgaacacccggctgctgac 3'

6)olfhnhum24.seq, bases 1677-1779 of $F_{RSV}HN_{Piv3}$, inverse
complementary of mARN
5'gatggtgatctcgctgatgaacttccgcaggtcggacatctgctgggtcagggagatggggat
gtagttctgcacgtggctctggatggtcagcagccgggtg 3'

7)olfhnhum25.seq, bases 1761-1865 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'catcagcgagatcaccatccggaacgacaaccaggaggtgccccccccagaggatcacccacga
cgtgggcataaagcccctgaaccccgacgacttctggcgctg 3'

8)olfhnhum26.seq, bases 1849-1967 of $F_{RSV}HN_{Piv3}$, inverse
complementary of mARN
5'gtgcgcacgcagccgtccacggtggtgggcatggccagcaggccgggcccgggcatcagcctt
atcttgggggtcttcatcagggaggggaggccggaggtgcagcgccagaagtcgtc 3'

9)olfhnhum27.seq, bases 1953-2059 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'cggctgcgtgcgcacccccctccctggtgatcaacgacctgatctacgcctacacctccaacct
gatcacccgcggctgccaggacatcggcaagtcctaccaggtgc 3'

10)olfhnhum28.seq, bases 2043-2154 of $F_{RSV}HN_{Piv3}$,, inverse
complementary of mARN
5'ggacttcctgttgtcgttgatgttgaaggtgtgggagatccgggggttcaggtcgggcaccag
gtcggagttcacggtgatgatgccgatctgcagcacctggtaggacttg

[SEQUENCES ABOVE ARE SEQ ID NOs: 30-39, respectively]

Fig. 31: Sequence of the 16 oligonucleotides from which PCR fragment C was generated.

1)olfhnum29.seq, bases 2139-2229 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'cgacaacaggaagtcctgctccctggccctcctgaacaccgacgtgtaccagctgtgctccac
gcccaaggtggacgagcgctccgactac 3'

2)olfhnhum30.seq, bases 2214-2307 of $F_{RSV}HN_{PiV3}$, inverse
complementary to mRNA
5'gttcttgaagcgggtggtggagatggagccgtcgtggttgacgatgtccagcacgatgtcctc
gatgccggagctggcgtagtcggagcgctcg 3'

3)olfhnhum31.seq, bases 2292-2398 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'cacccgcttcaagaacaacaacatcagcttcgaccagccctacgccgccctgtacccctccgt
gggccccggcatctactacaagggcaagatcatcttcctgggc 3'

4)olfhnhum32.seq, bases 2382-2472 of $F_{RSV}HN_{PiV3}$, inverse
complementary to mRNA
5'ccgctgggtcttgccggggcacccggtggtgttgcagatggcgttctcgttgatggggtgctc
caggccgccgtagcccaggaagatgatc 3'

5)olfhnhum33.seq, bases 2457-2549 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'cggcaagacccagcgggactgcaaccaggcctcccacagcccctggttctccgaccgccgcat
ggtgaactccatcatcgtggtggacaaggg 3'

6)olfhnhum34.seq, bases 2532-2643 of $F_{RSV}HN_{PiV3}$, inverse
complementary to mRNA
5'cttgttgcccagcagcagcaggcggccctcggagccccagtagttctgccgcatggagatggt
ccacaccttcagcttggggatggagttcaggcccttgtccaccacgatg 3'

7)olfhnhum35.seq, bases 2628-2726 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'gctgctgggcaacaagatctacatctacacccgctccaccagctggcacagcaagctgcagct
gggcatcatcgacatcaccgactacagcgacatccg 3'

8)olfhnhum36.seq, bases 2710-2781 of $F_{RSV}HN_{PiV3}$, inverse
complementary to mRNA
5'ggggcactcgttgttgccgggccggctcagcacgttgtgccaggtccacttgatgcggatgtc
gctgtagtc 3'

9)olfhnhum37.seq, bases 2765-2836 of $F_{RSV}HN_{PiV3}$, homologous to mRNA
5'gcaacaacgagtgcccctggggccactcctgccccgacggctgcatcaccggcgtgtacaccg
acgcctacc 3'

10)olfhnhum38.seq, bases 2820-2889 of $F_{RSV}HN_{PiV3}$, inverse
complementary to mRNA
5'cttctgggagtccaggatcacggagctcacgatgctgccggtggggttcaggggtaggcgtc
ggtgtac

11)olfhnhum39.seq, bases 2874-2943 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'cctggactcccagaagtcccgggtgaaccccgtgatcacctacagcacctccaccgagcgcgt
gaacgag

12)olfhnhum40.seq, bases 2927-2994 of $F_{RSV}HN_{Piv3}$from: 1 to: 68,
inverse complementary to mRNA
5'gcagctggtggtggtgtagccggcgctcagggtcttgttgcggatggccagctcgttcacgcg
ctcgg

13)olfhnhum41.seq,bases 2979-3043 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'caccaccaccagctgcatcacccactacaacaagggctactgcttccacatcgtggagatcaa
cc

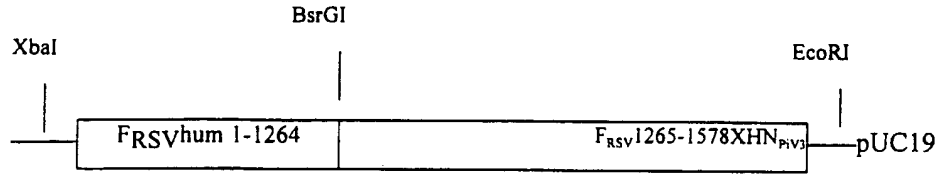
14)olfhnhum42.seq, bases 3027-3085 of $F_{RSV}HN_{Piv3}$, inverse
complementary to mRNA
5'cggtcttgaacagcatgggctggaaggtgtccaggctcttgtggttgatctccacgatg 3'

15)olfhnhum43.seq, bases 3069-3114 of $F_{RSV}HN_{Piv3}$, homologous to mRNA
5'catgctgttcaagaccgagatccccaagagctgcagctaaGAATTC 3'

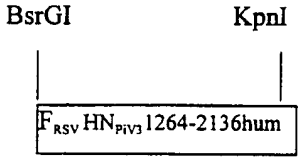
[SEQUENCES ABOVE ARE SEQ ID NOs : 40-54, respectively]

Fig. 32 : Construction of pEE14F_{RSV}hum HN $_{PiV3}$hum

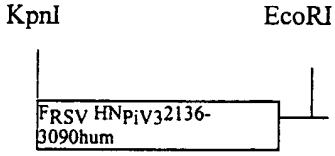
a) pNIV4120 +PCR fragment A



b) PCR fragment B



c) PCR fragment C



d) pEE14 F_{RSV}hum HN_{PiV3} hum

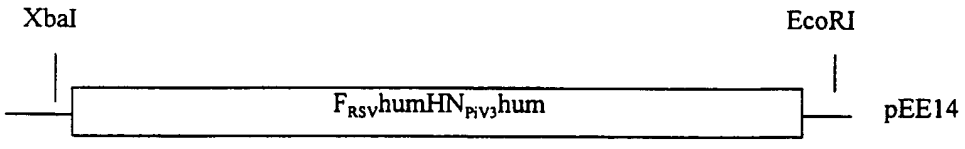


Fig. 33A : Humanized nucleic acids sequence of $F_{RSV}HN_{PIV3}$ (upper sequence) compared to the original sequence found in the pNIV4120.

```
   7 AGAGGATCC.......ACCATGGAGCTGCTGATtttaAAGACCAACGCCA 49
     |||||||||       |||||||||  ||||  ||   |  ||  ||  ||  ||  |
2262 AGAGGATCCCCCGGGTAccatggagttgctaatcctcaaaacaaatgcaa 2311
            .         .         .         .         .
  50 TCACCGCCATCCTGGCCGCGGTGACCCTCTGCTTCGCGTCCAGCCAGAAC 99
     | |||||  |||||  ||  ||  ||  ||  |||||  ||  ||  |||||  ||  |||
2312 ttaccgcaatccttgctgcagtcacactctgttttgcttccagtcaaaac 2361
            .         .         .         .         .
 100 ATCACCGAGGAGTTCTACCAGTCCACCTGCtctGCTGTCAGCAAGGGCTA 149
     |||||  ||  ||  ||  ||  ||  ||  |||   |||  ||||||||  |||||
2362 atcactgaagaattttatcaatcaacatgcagtgcagtcagcaaaggcta 2411
            .         .         .         .         .
 150 CCTGAGCGCGCTGAGGACGGGGTGGTACACtAGtGTGATCACCATCGAGC 199
      ||  ||  ||  ||  ||  ||  ||  |||||  ||||||||  ||  ||  ||  ||
2412 tcttagtgctctaagaactggttggtatactagtgttataactatagaat 2461
            .         .         .         .         .
 200 TGAGCAACATCAAGGAGAACAAGTGCAACGGCACCGACGCCAAGGTGAAG 249
     |  ||  ||  ||||||||  ||  |||||  ||  ||  ||  |||||  |||||  ||
2462 taagtaatatcaaggaaaataagtgtaatggaacagacgctaaggtaaaa 2511
            .         .         .         .         .
 250 CTGATCAAGCAGGAGCTGGACAAGTACAAGAGCGCCGTGACCGAGCTGCA 299
      ||||  ||  ||  ||   |  ||  ||  ||  ||  ||  ||  ||  ||   ||||
2512 ttgataaaacaagaattagataaatataaaagtgctgtaacagaattgca 2561
            .         .         .         .         .
 300 GCTGCTGATGCAGAGCACCCCCGCCACCAACAACagaGCCAGGCGCGAGC 349
     |  ||||  |||||  |||||  ||  ||  ||||||||   |||||||   |  ||  |
2562 gttgctcatgcaaagcacaccggcaaccaacaatcgagccagaagagaac 2611
            .         .         .         .         .
 350 TGCCCAGGTTCATGAACTACACCCTCAACAACACCAAGAACACCAACGTG 399
     |  ||  |||||  |||||  ||  ||  ||||||||  |||||  ||  |||||  ||
2612 taccaaggtttatgaattatacactcaacaataccaaaaataccaatgta 2661
            .         .         .         .         .
 400 ACCCTGAGCAAGAAGcGcAAGaggCGcTTCCTGGGCTTCCTGCTGGGCGT 449
     ||   |  ||||||||   |  ||  ||   |  ||  ||  |||||   ||  |  ||  ||
2662 acattaagcaagaaaaggaaaagaagatttcttggctttttgttaggtgt 2711
            .         .         .         .         .
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2712 tggatctgcaatcgccagtggcattgctgtatctaaggtcctgcacctag 2761
            .         .         .         .         .
 500 AGGGGGAGGTGAACAAGATCAAGAGCGCCCTGCTCTCCACCAACAAGGCG 549
     |  |||||  ||||||||  |||||  ||  ||  ||  ||  |||||  ||||||||
2762 aaggggaagtgaacaaaatcaaaagtgctctactatccacaaacaaggct 2811
            .         .         .         .         .
```

```
 550 GTGGTCAGCCTGTCCAACGGCGTGAGCGTGCTGACCAGCAAGGTGCTGGA 599
     || |||||| | || || || || || ||  | |||||||| ||| | ||
2812 gtagtcagcttatcaaatggagttagtgtcttaaccagcaaagtgttaga 2861
           .         .         .         .         .
 600 CCTCAAGAACTACATCGACAAGCAatTGCTCCCCATCGTGAACAAGCAGt 649
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 750 CACCCCGGTGAGCACCTACATGCTGACCAACAGCGAGCTGCTGTCCCTGA 799
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     |||| || ||||| || || || || ||||| ||| | ||||||||||
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           .         .         .         .         .
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     || || || ||  | ||||| || |||   ||||||  ||| || |||||
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 900 GGAGGTGCTGGCCTACGTGGTGCAGCTGCCCCTGTACGGCGTGATCGACA 949
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3162 ggaagtcttagcatatgtagtacaattaccactatatggtgtaatagata 3211
           .         .         .         .         .
 950 CCCCCTGCTGGAAGCTGCACACCTCCCCCCTGTGCACCACCAACACCAAG 999
     | || || ||||| |||||||| ||||| || || || |||||||| |||
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           .         .         .         .         .
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     ||| || || || || || |||||||| || || || || || || || |
3312 caatgcaggatcagtatctttcttcccactagctgaaacatgtaaagttc 3361
           .         .         .         .         .
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1200 GATtATGacctccaagaccgacgtgagcagctccgtgatcacctccctgg 1249
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3762 agtcaatgagaagattaaccagagcctagcatttattcgtaaatccgatg 3811
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4012 CTTAGGAAATTCATTAGTGAAATTACAATTAGGAATGATAATCAAGAAGT 4061
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1800 gccccccagaggatcacccacgacgtgggcataaagcccctgaaccccg 1849
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1850 acgacttctggcgctgcacctccggcctcccctccctgatgaagacccccc 1899
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1900 aagataaggctgatgcccgggcccggcctgctggccatgcccaccaccgt 1949
     ||  ||||||  |  |||||  |||||  ||   |   |  ||  |||||  ||  ||  ||
4162 AAAATAAGGTTAATGCCGGGGCCGGGATTATTAGCTATGCCAACGACTGT 4211
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1950 ggacggctgcgtgcgcacccccctccctggtgatcaacgacctgatctacg 1999
      ||  |||||  ||   |  ||  ||  |||  |  ||  ||  ||  ||  |||||  ||  |
4212 TGATGGCTGTGTTAGAACTCCGTCCTTAGTTATAAATGATCTGATTTATG 4261
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2000 cctacacctccaacctgatcacccgcggctgccaggacatcggcaagtcc 2049
     |  ||  |||||  ||  ||  ||  ||  ||  ||  ||||||||  ||  ||  ||  ||
4262 CTTATACCTCGAATCTAATTACTCGAGGTTGCCAGGATATAGGAAAATCA 4311
                .         .         .         .         .
2050 taccaggtgctgcagatcggcatcatcaccgtgaactccgacctggtacc 2099
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                .         .         .         .         .
2100 cgacctgaacccccggatctcccacaccttcaacatcaacgacaacagga 2149
      |||  |  ||  ||   |||||||  ||  ||  ||||||||  ||  |||||  ||  |
4362 TGACTTAAATCCTAGGATCTCTCATACTTTCAACATAAATGACAATAGAA 4411
                .         .         .         .         .
2150 agtcctgctccctggccctcctgaacaccgacgtgtaccagctgtgctcc 2199
     ||||  ||  ||  ||  ||  |||||  |||||  ||  ||  ||  ||  |||||  ||
4412 AGTCATGTTCTCTAGCACTCCTAAACACAGATGTATATCAACTGTGTTCG 4461
                .         .         .         .         .
2200 acgcccaaggtggacgagcgctccgactacgccagctccggcatcgagga 2249
     ||  |||||  ||  ||  ||   |  ||  ||  ||  ||    ||  |||||  ||  ||
4462 ACTCCCAAAGTTGATGAAAGATCAGATTATGCATCATCAGGCATAGAAGA 4511
                .         .         .         .         .
2250 catcgtgctggacatcgtcaaccacgacggctccatctccaccacccgct 2299
      ||  ||  ||  ||  ||  |||||  ||  ||  ||  ||  |||||  ||  ||   |  |
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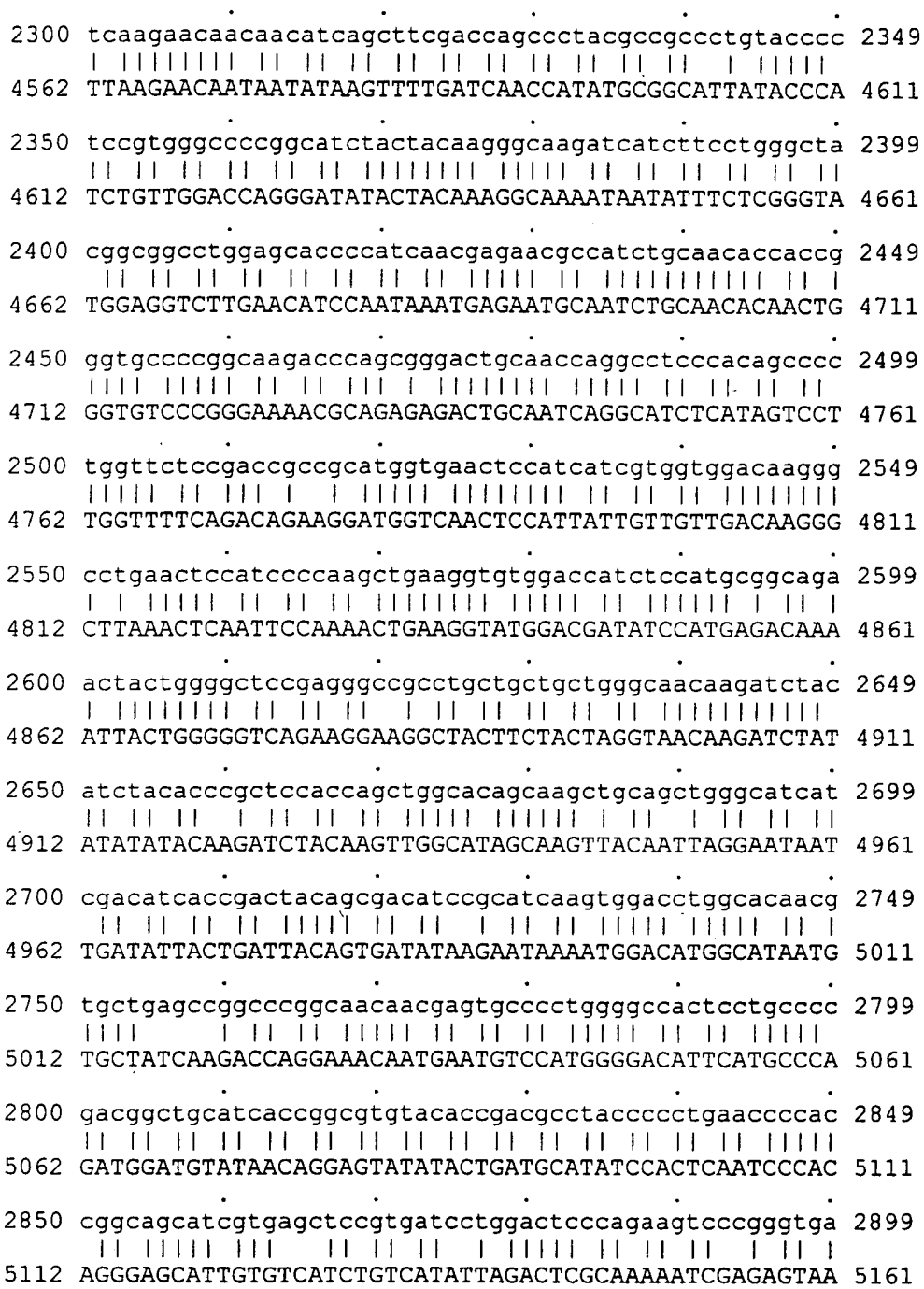

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5162 ACCCAGTCATAACTTACTCAACATCAACTGAAAGGGTAAACGAGCTGGCC 5211
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2950 atccgcaacaagaccctgagcgccggctacaccaccaccagctgcatcac 2999
     |||||  |||||  ||  ||     ||  ||  ||  ||  ||  ||  ||||||||  ||
5212 ATCCGAAACAAAACACTCTCAGCTGGATATACAACAACGAGCTGCATTAC 5261
                .         .         .         .         .
3000 ccactacaacaagggctactgcttccacatcgtggagatcaaccacaaga 3049
      |||||  |||||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  ||  |
5262 ACACTATAACAAAGGATATTGTTTTCATATAGTAGAAATAAATCATAAAA 5311
                .         .         .         .         .
3050 gcctggacaccttccagcccatgctgttcaagaccgagatccccaagagc 3099
     ||  |  |||||  |||||  ||  |||  |||||||  ||  |||||  ||  ||  |||
5312 GCTTAGACACATTCCAACCTATGTTGTTCAAAACAGAGATTCCAAAAAGC 5361
                .
3100 tgcagctaaGAAT 3112
     |||||  |||   ||
5362 TGCAGTTAATCAT 5374
```

Fig. 33B

(Linear) MAP of: FrsvHNpiv3.seq check: 7448 from: 1 to: 3090 nucleic acids sequence of FrsvHNpiv3 (non humanised)

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aatcaacatgcagtgcagtcagcaaaggctatcttagtgctctaagaact

ggttggtatactagtgttataactatagaattaagtaatatcaaggaaaa

taagtgtaatggaacagacgctaaggtaaaattgataaaacaagaattag

ataaatataaaagtgctgtaacagaattgcagttgctcatgcaaagcaca

ccggcaaccaacaatcgagccagaagagaactaccaaggtttatgaatta

tacactcaacaataccaaaaataccaatgtaacattaagcaagaaaagga

aaagaagatttcttggctttttgttaggtgttggatctgcaatcgccagt

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AAATTACAATTAGGAATGATAATCAAGAAGTGCCTCCACAAAGAATAACA

CATGATGTGGGCATAAAACCTTTAAATCCAGATGATTTTTGGAGATGCAC

GTCTGGTCTTCCATCTTTAATGAAAACTCCAAAAATAAGGTTAATGCCGG

GGCCGGGATTATTAGCTATGCCAACGACTGTTGATGGCTGTGTTAGAACT

CCGTCCTTAGTTATAAATGATCTGATTTATGCTTATACCTCaAATCTAAT

TACTCGAGGTTGCCAGGATATAGGAAAATCATATCAAGTATTACAGATAG

GGATAATAACTGTAAACTCAGACTTGGTACCTGACTTAAATCCTAGGATC

TCTCATACTTTCAACATAAATGACAATAGAAAGTCATGTTCTCTAGCACT

CCTAAAtACAGATGTATATCAACTGTGTTCGACTCCCAAAGTTGATGAAA

GATCAGATTATGCATCATCAGGCATAGAAGATATTGTACTTGATATtGTC

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TGGTCAACTCCATTATTGTTGTTGACAAGGGCTTAAACTCAATTCCAAAA

CTGAAGGTATGGACGATATCCATGAGACAAAATTACTGGGGGTCAGAAGG

AAGGCTACTTCTACTAGGTAACAAGATCTATATATATACAAGATCTACAA

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CAGCTGGATATACAACAACGAGCTGCATTACACACTATAACAAAGGATAT

TGTTTTCATATAGTAGAAATAAATCATAAAAGCTTAGACACATTCCAACC

TATGTTGTTCAAAACAGAGATTCCAAAAAGCTGCAGTTAA

[SEQ ID NO: 55]

Fig. 33C

(Linear) MAP of: FrhumHNphum.seq check: 9920 from: 1 to: 3090 Humanised nucleic acids sequence of FRSVHNPiV3

GGGTGGTACACtAGtGTGATCACCATCGAGCTGAGCAACATCAAGGAGAA

CAAGTGCAACGGCACCGACGCCAAGGTGAAGCTGATCAAGCAGGAGCTGG

ACAAGTACAAGAGCGCCGTGACCGAGCTGCAGCTGCTGATGCAGAGCACC

CCCGCCACCAACAACagaGCCAGGCGCGAGCTGCCCAGGTTCATGAACTA

CACCCTCAACAACACCAAGAACACCAACGTGACCCTGAGCAAGAAGcGcA

AGaggCGcTTCCTGGGCTTCCTGCTGGGCGTGGGCTCCGCCATCGCCAGC

GGCATCGCGGTGTCCAAGGTCCTGCACCTGGAGGGGGAGGTGAACAAGAT

CAAGAGCGCCCTGCTCTCCACCAACAAGGCGGTGGTCAGCCTGTCCAACG

GCGTGAGCGTGCTGACCAGCAAGGTGCTGGACCTCAAGAACTACATCGAC

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CCAGGGAGTTCAGCGTGAACGCgGGcGTcACCACCCCGGTGAGCACCTAC

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GTGCAGCTGCCCCTGTACGGCGTGATCGACACCCCCTGCTGGAAGCTGCA

CACCTCCCCCCTGTGCACCACCAACACCAAGGAGGGCTCCAACATCTGCC

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[SEQ ID NO : 56]

Fig. 34A

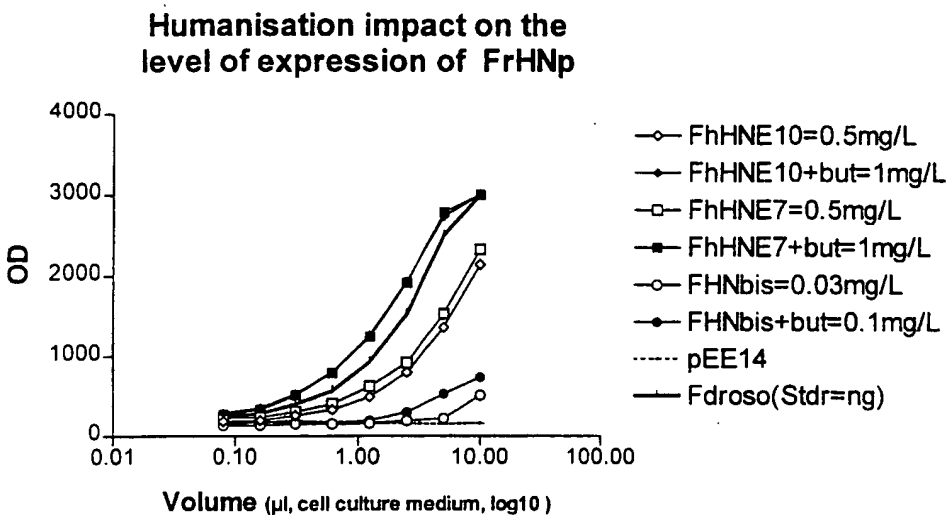

Humanisation impact on the level of expression of FrHNp
OD
4000
3000
2000
1000
0
0.01
0.10
1.00
10.00
100.00
Volume (µl, cell culture medium, log10)
FhHNE10=0.5mg/L
FhHNE10+but=1mg/L
FhHNE7=0.5mg/L
FhHNE7+but=1mg/L
FHNbis=0.03mg/L
FHNbis+but=0.1mg/L
pEE14
Fdroso(Stdr=ng)

Fig. 34B: Humanization impact on the level of expression of $F_{RSV}HN_{PiV3}$

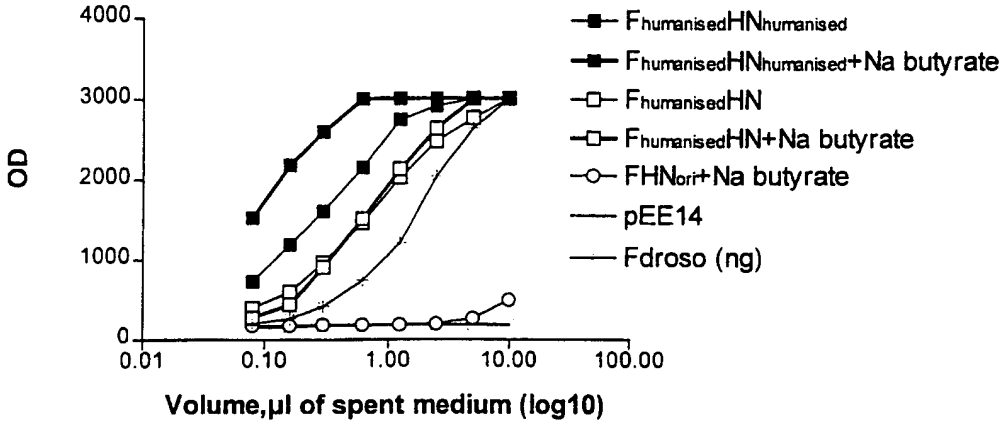


Fig 35: Codon usage of $F_{MuV}H_{MV}$ and highly expressed human genes (hum high exp)

The frequencies (x100) of the individual codons are shown for each of the degenerately encoded amino acids, and the most prevalent codon is shown in bold.

		hum high exp	$F_{MuV}H_{MV}$
Ala	GCG	17	10
	A	13	**47**
	T	17	25
	C	**53**	18
Arg	AGG	18	**30**
	A	10	**30**
	CGG	21	10
	A	6	15
	T	7	8
	C	**37**	8
Asn	AAT	22	**60**
	C	**78**	40
Asp	GAT	25	**59**
	C	**75**	41
Cys	TGT	32	**56**
	C	**68**	44
Gln	CAG	**88**	32
	A	12	**68**
Glu	GAG	**75**	52
	A	25	**48**
Gly	GGG	24	24
	A	14	26
	T	12	**33**
	C	**50**	17
His	CAT	21	**60**
	C	**79**	40
Ile	ATA	5	25
	T	18	35
	C	**77**	**39**
Leu	TTG	6	**25**
	A	2	11
	CUG	**58**	15
	A	3	20
	T	5	14
	C	26	15
Lys	AAG	**82**	**51**
	A	18	49
Phe	TTT	20	39
	C	**80**	**61**
Pro	CCG	17	19
	A	16	**40**
	T	19	23
	C	**48**	19
Ser	AGT	10	19
	C	**34**	15
	TCG	9	5
	A	5	**30**
	T	13	17
	C	28	14
Thr	ACG	15	7
	A	14	**41**
	T	14	30
	C	**57**	21
Tyr	TAT	26	34
	C	**74**	**66**
Val	GTG	**64**	**34**
	A	5	10
	T	7	27
	C	25	29

Fig. 36: Schematic diagram of the PCR synthesis of each fragment in which X and Y are restriction sites that allow retrieval of the full size fragment from the cloning vector.

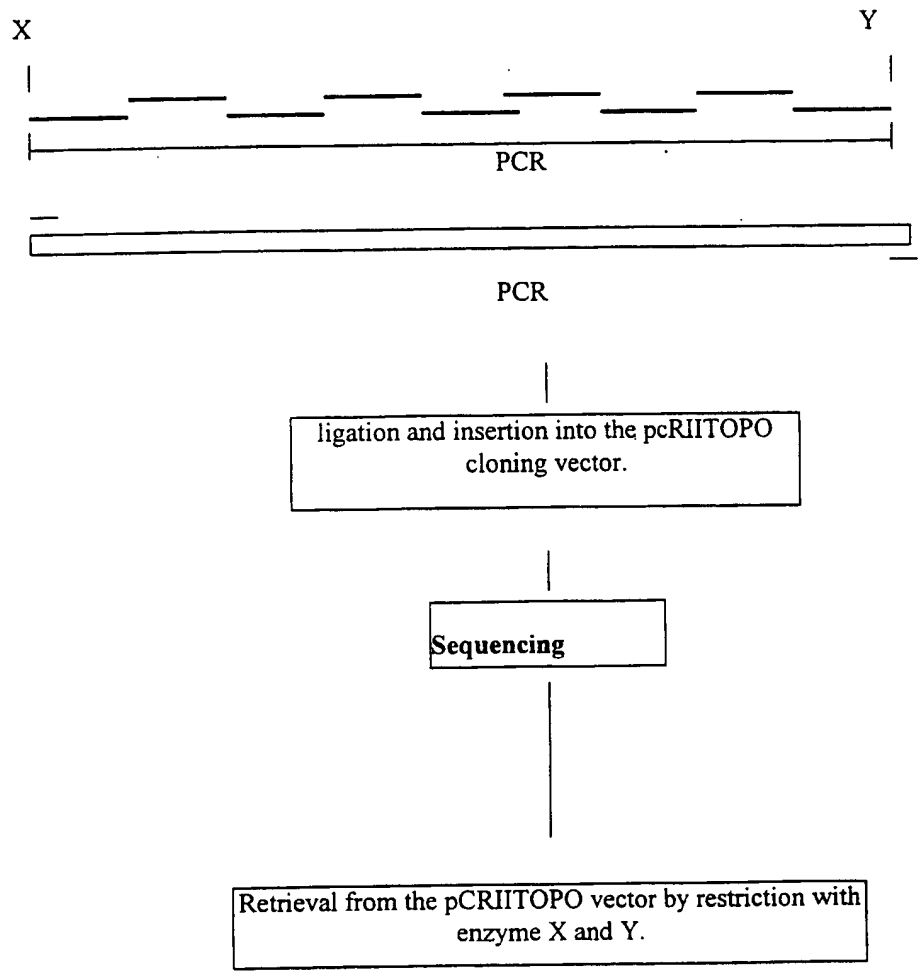


Fig 37 : Sequence of the 12 oligonucleotides from which PCR fragment A was generated.

```
1)oli 1 FmuvHmv 1-98,hom ARN
ggtctagaccaccATGAAGGCGTTCCCCGTGATCTGCCTGGGCTTCGCCATCTTCTCCAG
---------+---------+---------+---------+---------+---------+ 60
CAGCATCTGCGTGAACATCAACATCCTGCAGCAGATCG
---------+---------+---------+-------- 98


2) oli 2 FmuvHmv 82-181,inv comp ARN
GTTGGGCAGCAGCTTGACCACCACGTAGGAGCTGGAGCTCTGGGAGTAGTAGCTCAGCTG
---------+---------+---------+---------+---------+---------+ 60
CCTCACCTGCTGCTTGATGTATCCGATCTGCTGCAGGATG
---------+---------+---------+---------+ 100


3) oli 3 FmuvHmv, 166-264 hom ARN
CAAGCTGCTGCCCAACATCCAGCCCACCGACAACAGCTGCGAGTTCAAGAGCGTGACCCA
---------+---------+---------+---------+---------+---------+ 60
GTACAACAAGACCCTGAGCAACCTGCTGCTGCCCATCGC
---------+---------+---------+--------- 99


4) oli 4 FmuvHmv, 250-352, inv compARN
CAGGGCGGCGATGCCGATGGCGATGCCGGCGAACCGCTTGTGCCGCCGGGAGCCGGGGGA
---------+---------+---------+---------+---------+---------+ 60
GGGGGAGGTGATGTTGTTGATGTTCTCGGCGATGGGCAGCAGC
---------+---------+---------+---------+--- 103


5) oli 5 FmuvHmv, 338-441, hom ARN
GGCATCGCCGCCCTGGGCGTGGCCACCGCCGCCCAGGTGACCGCCGCCGTGTCCCTGGTG
---------+---------+---------+---------+---------+---------+ 60
CAGGCCCAGACCAACGCCCGCGCCATCGCCGCCATGAAGAACTC
---------+---------+---------+---------+---- 104


6) oli 6 FMuvHmv, 427-523, inv comp ARN
GTCCTGGATGGCCTGCACGGCGATGGCCAGCTGCTGGGTGCCCTCCTTCACCTCGAACAC
---------+---------+---------+---------+---------+---------+ 60
GGCGCGGTTGGTGGCCTGGATGGAGTTCTTCATGGCG
---------+---------+---------+------- 97


7) oli 7 FMUVHM 509-610, hom ARN
CAGGCCATCCAGGACCACATCAACACCATCATGAACACCCAGCTGAACAACATGTCCTGC
---------+---------+---------+---------+---------+---------+ 60
CAGATCCTGGACAACCAGCTGGCCACCTCCCTGGGCCTGTAC
---------+---------+---------+---------+-- 102
```

8) oli 8 FMUHM, 595-691, inv comp ARN
GGACCGCAGGGCCTGGATACTGATGGGggaCAGGGCGGGGTTGATCAGCTGGGGCTGGAA
---------+---------+---------+---------+---------+---------+ 60
CACGGTGGTCAGCTCGGTCAGGTACAGGCCCAGGGAG
---------+---------+---------+------- 97

9) oli 9, 677-778, hom ARN
CAGGCCCTGCGGTCCCTGCTGGGCAGCATGACCCCCGCCGTGGTGCAGGCCACCCTGAGC
---------+---------+---------+---------+---------+---------+ 60
ACCTCCATCAGCGCCGCCGAGATCCTGAGCGCCGGCCTGATG
---------+---------+---------+---------+-- 102

10) oli 10, FmuvHmv, 763-862, inv comp ARN
GTTGGACTGGGTCACGATGGTGGGCACGTTGATCTTCACGATCATCTGCATCTCGTCCAG
---------+---------+---------+---------+---------+---------+ 60
CAGCACGGACACGATCTGGCCCTCCATCAGGCCGGCGCTC
---------+---------+---------+---------+ 100

11) oli 11, FmuvHmv, 848-949, homARN
GTGACCCAGTCCAACGCCCTGGTGATCGACTTCTACAGCATCAGCAGCTTCATCAACAAC
---------+---------+---------+---------+---------+---------+ 60
CAGGAGTCCATCATCCAGCTGCCCGACCGCATCCTGGAGATC
---------+---------+---------+---------+-- 102

12) oli 12 FMUHM, 935-1039, inv compARN
GCTCAGCCGCTCGGCCTCGTTGTACTGGCAGAAGATGTGGTGGCGGGTCAGCTTGCAGTT
---------+---------+---------+---------+---------+---------+ 60
CTTGGCGGGGTAGCGCCACTGCTCGTTGCCGATCTCCAGGATGCG
---------+---------+---------+---------+----- 105

[SEQ ID NOS: 57-68 respectively]

Fig. 38 : Sequence of the 9 oligonucleotides from which PCR fragment B was generated.

```
12) oli 12 FMUHM, 935-1039, inv compARN
GCTCAGCCGCTCGGCCTCGTTGTACTGGCAGAAGATGTGGTGGCGGGTCAGCTTGCAGTT
---------+---------+---------+---------+---------+---------+ 60
CTTGGCGGGGTAGCGCCACTGCTCGTTGCCGATCTCCAGGATGCG
---------+---------+---------+---------+----- 105


13) oli 13, 1025-1129, hom ARN
GCCGAGCGGCTGAGCCTGGAGACCAAGCTGTGCCTGGCCGGCAACATCAGCGCCTGCGTG
---------+---------+---------+---------+---------+---------+ 60
TTCTCCAGCATCGCCGGCAGCTACATGCGCCGCTTCGTGGCCCTG
---------+---------+---------+---------+----- 105


14) oli 14, 1115-1216, inv comp ARN
GGCGTGGTGGTCGGGCTGGTAGATGGGGTAGGAGGGGCTCTTGCACAGGCAGGTCAGGCT
---------+---------+---------+---------+---------+---------+ 60
GCGGCAGTTGGCCACGATGGTGCCGTCCAGGGCCACGAAGCG
---------+---------+---------+---------+-- 102


15) oli 15, 1202-1299, hom ARN
CCCGACCACCACGCCGTGACCACCATCGACCTGACCTCCTGCCAGACCCTGAGCCTGGAC
---------+---------+---------+---------+---------+---------+ 60
GGCCTGGACTTCAGCATCGTGTCCCTGAGCAACATCAC
---------+---------+---------+-------- 98


16) oli 16 1285-1387, inv comp ARN
CTTGCTCAGCTCGGTGGAGATGTCGATGGGCTGGGTGTTGATGGTCTGGCTCAGGCTGAT
---------+---------+---------+---------+---------+---------+ 60
GGTCAGGTTCTCGGCGTAGGTGATGTTGCTCAGG
---------+---------+---------+---- 94


17) oli 17, 1363-1462, hom ARN
CACCGAGCTGAGCAAGGTGAACGCCTCCCTGCAGAACGCCGTGAAGTACATCAAGGAGAG
---------+---------+---------+---------+---------+---------+ 60
CAACCACCAGCTGCAGAGCGTGAGCGTGAGCAGCAAGCGC
---------+---------+---------+---------+ 100


18) oli 18, 1447-1550, inv comp ARN
TCACCTGGTGCTCGATGGAGTTGGTCACGTCCAGGTTGGTGCTCAGGCTCTTGTGGATCT
---------+---------+---------+---------+---------+---------+ 60
CGGCGGTGTAGATGGCGGCGCGGTGCAGGCGCTTGCTGCTCACG
---------+---------+---------+---------+---- 104
```

```
19) oli 19, 1534-1636, hom ARN
CATCGAGCACCAGGTGAAGGACGTGCTGACCCCCCTGTTCAAGATCATCGGCGACGAGGT
---------+---------+---------+---------+---------+---------+ 60
GGGCCTGCGCACCCCCCAGCGCTTCACCGACCTGGTGAAGTTC
---------+---------+---------+---------+--- 103


20) oli 20 FmuvHmv, 1622-1718, inv comp ARN
GCTCGGGGGGGTTGATGCACCAGGTCAGGTCGCGGAAGTCGTACTCGCGGTCGGGGTTCA
---------+---------+---------+---------+---------+---------+ 60
GGAACTTGATCTTGTCGGAGATGAACTTCACCAGGTC
---------+---------+---------+------- 97
```

[SEQ ID NOS: 69-77 respectively]

Fig. 39 : Sequence of the 11 oligonucleotides from which PCR fragment C was generated.

```
20) oli 20 FmuvHmv, 1622-1718, inv comp ARN
GCTCGGGGGGGTTGATGCACCAGGTCAGGTCGCGGAAGTCGTACTCGCGGTCGGGGTTCA
---------+---------+---------+---------+---------+---------+ 60
GGAACTTGATCTTGTCGGAGATGAACTTCACCAGGTC
---------+---------+---------+------- 97

21) oli 21, FmuvHmv, 1701-1799, hom ARN
GCATCAACCCCCCCGAGCGGATCAAGCTGGACTACGACCAGTACTGCGCCGACGTGGCCG
---------+---------+---------+---------+---------+---------+ 60
CCGAGGAGCTGATGAACGCCCTGGTGAACAGCACCCTGC
---------+---------+---------+--------- 99

22) oli 22,1784-1888, inv comp
CATGTTGCTGAACTGGCCCCGGATGGTGGTGGGGCCGCTGCAGTTGCCCTTGCTCACGGC
---------+---------+---------+---------+---------+---------+ 60
CAGGAACTGGTTGGTGGTGCGGGTCTCCAGCAGGGTGCTGTTCAC
---------+---------+---------+---------+----- 105

23) oli 23, 1874-1971, hom ARN
CAGTTCAGCAACATGAGCCTGTCCCTGCTGGACCTGTACCTGGGCCGGGGCTACAACGTG
---------+---------+---------+---------+---------+---------+ 60
AGCAGCATCGTGACCATGACCAGCCAGGGCATGTACGG
---------+---------+---------+-------- 98

24) oli 24, 1957-2057, inv.comp ARN
CCACCTCGAACACGCGGTACATGCTCAGCTGGCTCAGCTCGCTCCGCTTGCTGCTCAGGT
---------+---------+---------+---------+---------+---------+ 60
TGGGCTTCTCCACCAGGTAGGTGCCGCCGTACATGCCCTGG
---------+---------+---------+---------+- 101

25) oli 25, FmuvHmv, 2043-2140, homARN
GCGTGTTCGAGGTGGGCGTGATCCGGAACCCCGGCCTGGGCGCCCCCGTGTTCCACATGA
---------+---------+---------+---------+---------+---------+ 60
CCAACTACCTGGAGCAGCCCGTGAGCAACGACCTGAGC
---------+---------+---------+-------- 98

26) oli26, FmuvHmv, 2125-2227, inv compARN
GCCGCTGCCCTGGTAGGGGATGGTGATGCTGTCCTCGCCGTGGCACAGGGCGGCCAGCTT
---------+---------+---------+---------+---------+---------+ 60
CAGCTCGCCCAGGGCCACCATGCAGTTGCTCAGGTCGTTGCTC
---------+---------+---------+---------+--- 103

27) oli 27, 2212-2309, FmuvHm, hom ARN
CTACCAGGGCAGCGGCAAGGGCGTGAGCTTCCAGCTGGTGAAGCTGGGCGTGTGGAAGAG
---------+---------+---------+---------+---------+---------+ 60
CCCCACCGACATGCAGAGCTGGGTGCCCCTGAGCACCG
---------+---------+---------+-------- 98
```

28) oli 28,FmuvHmv, 2294-2392, inv comp ARN

```
GGTGGGCACGGCCCACTTGGCCTGGTTGTCGGCGATCACGCCGCGGTGGCTGCTCAGGTA
---------+---------+---------+---------+---------+---------+ 60
CAGGCGGTCGATCACGGGGTCGTCGGTGCTCAGGGGCAC
---------+---------+---------+--------- 99
```

29) oli 29, Fmuv Hmv, 2377-2477, hom ARN

```
GTGGGCCGTGCCCACCACCCGCACCGACGACAAGCTGCGCATGGAGACCTGCTTCCAGCA
---------+---------+---------+---------+---------+---------+ 60
GGCCTGCAAGGGCAAGATCCAGGCCCTGTGCGAGAACCCCG
---------+---------+---------+---------+- 101
```

30) oli 30, FmuvHmv 2462-2561, inv comp

```
TGATCTTCAGCTCCACGGTCAGGCTCAGGTCCACGCTCAGCACGCCGTAGCTGGGGATGC
---------+---------+---------+---------+---------+---------+ 60
GGTTGTCCTTCAGGGGGGCCCAtTCGGGGTTCTCGCACAG
---------+---------+---------+---------+ 100
```

[SEQ ID NOS: 78-88 respecively]

Fig. 40 : Sequence of the 8 oligonucleotides from which PCR fragment D was generated.

```
30) oli 30, FmuvHmv 2462-2561, inv comp
TGATCTTCAGCTCCACGGTCAGGCTCAGGTCCACGCTCAGCACGCCGTAGCTGGGGATGC
---------+---------+---------+---------+---------+---------+ 60
GGTTGTCCTTCAGGGGGGCCCAtTCGGGGTTCTCGCACAG
---------+---------+---------+---------+ 100

31) oli31, FmuvHmv, 2546-2649, hom ARN
GTGGAGCTGAAGATCAAGATCGCGAGCGGCTTCGGCCCCCTGATCACCCACGGCAGCGGC
---------+---------+---------+---------+---------+---------+ 60
ATGGACCTGTACAAGAGCAACCACAACAACGTGTACTGGCTGAC
---------+---------+---------+---------+---- 104

32) oli 32, FmuvHmv, 2635-2738, inv comp ARN
CGGTGAACAGGTAGGGGCTCACCTTGAAGCGGGGaATCCACTCCAGGGTGTTGATCACGC
---------+---------+---------+---------+---------+---------+ 60
CCAGGGCCAGGTTCTTCATGGGGGGGATGGTCAGCCAGTACACG
---------+---------+---------+---------+---- 104

33) oli 33, FmuvHmv, 2723-2827, hom ARN
CCCTACCTGTTCACCGTGCCCATCAAGGAGGCCGGCGAGGACTGCCACGCCCCGACCTAC
---------+---------+---------+---------+---------+---------+ 60
CTGCCCGCCGAGGTGGACGGCGACGTGAAGCTGAGCAGCAACCTG
---------+---------+---------+---------+----- 105

34) oli 34, FMUVHmv, 2813-2911, inv comp ARN
CACGTAGTACACCACGGCGTGCTCCACGCGGCTGGTGTCGTAGGTGGCCAGCACGTACTG
---------+---------+---------+---------+---------+---------+ 60
CAGGTCCTGGCCGGGCAGGATCACCAGGTTGCTGCTCAG
---------+---------+---------+--------- 99

35) oli 35 FMUHM, 2897-2995, homARN
GTGGTGTACTACGTGTACAGCCCCGGCCGCAGCTTCTTCTACTTCTACCCCTTCCGCCTG
---------+---------+---------+---------+---------+---------+ 60
CCCATCAAGGGCGTGCCCATCGAGCTGCAGGTGGAGTGC
---------+---------+---------+--------- 99

36) oli 36, FmuvHmv, 2981-3078, inv comp ARN
CCGCTGTGGGTGATGTGGCCGCCGCTCTCGCTGTCGGCCAGCACGCAGAAGTGGCGGCAC
---------+---------+---------+---------+---------+---------+ 60
CACAGCTTCTGGTCCCAGGTGAAGCACTCCACCTGCAG
---------+---------+---------+-------- 98
```

37) oli 37, 3064-3147, homARN

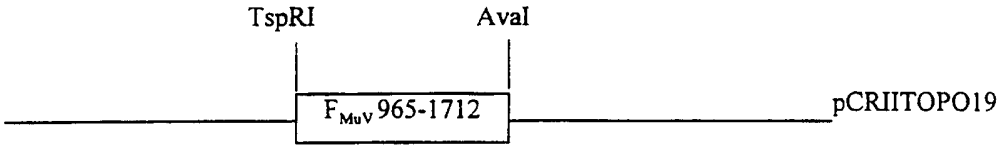
```
CATCACCCACAGCGGCATGGTGGGCATGGGCGTGAGCTGCACCGTGACCCGCGAGGACGG
---------+---------+---------+---------+---------+---------+ 60
CACCAACCGCCGCTAGcgaattcc
---------+---------+---- 84
```

[SEQ ID NOS: 89-96 respectively]

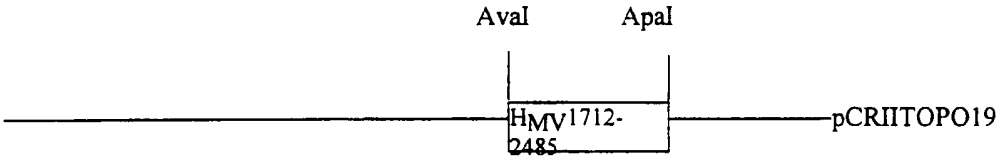
Fig. 41 : Construction of pEE14F_{MuV}hum HN_{MV} hum

a) PCR fragment A

b)pCR fragment B



c) PCR fragment C



d) PCR fragment D

d) pEE14 F_{MuV}hum H_{MV} hum

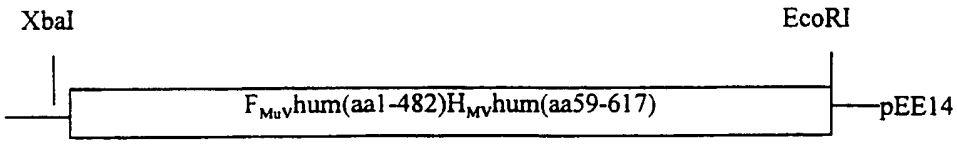


Figure 42A : Humanised nucleic acids sequence of $F_{MuV}H_{MV}$ (upper sequence) compared to the original $F_{MuV}H_{MV}$ sequence (lower sequence) and the corresponding amino acids sequence.

```
  14 ATGAAGGCGTTCCCCGTGATCTGCCTGGGCTTCGCCATCTTCTCCAGCAG 63
     ||||||||  ||  ||  ||  ||  |||  |||||||  ||  |||||  ||    |
   1 ATGAAGGCTTTTCCAGTTATTTGCTTGGGCTTTGCAATCTTTTCATCCTC 50

  64 CATCTGCGTGAACATCAACATCCTGCAGCAGATCGGATACATCAAGCAGC 113
      ||  ||  |||||  |||||  |||  |||||||  ||  ||||||||||||||  |
  51 TATATGTGTGAATATCAATATCTTGCAGCAAATTGGATACATCAAGCAAC 100

 114 AGGTGAGGCAGCTGAGCTACTACTCCCAGAGCTCCAGCTCCTACGTGGTG 163
     ||||  |||||  ||  |||||  |||||  ||  ||  ||  ||||||||||||  |||
 101 AGGTCAGGCAACTAAGCTATTACTCACAAAGTTCAAGCTCCTACGTAGTG 150

 164 GTCAAGCTGCTGCCCAACATCCAGCCCACCGACAACAGCTGCGAGTTCAA 213
     ||||||||   |  ||  ||  |||||  |||||  ||  ||||||||  ||  ||  ||
 151 GTCAAGCTTTTACCGAATATCCAACCCACTGATAACAGCTGTGAATTTAA 200

 214 GAGCGTGACCCAGTACAACAAGACCCTGAGCAACCTGCTGCTGCCCATCG 263
     |||  ||  ||  ||  |||||  ||||||  ||||  ||   ||||  ||  ||  ||  |
 201 GAGTGTAACTCAATACAATAAGACCTTGAGTAATTTGCTTCTTCCAATTG 250

 264 CCGAGAACATCAACAACATCACCTCCCCCTCCCCCGGCTCCCGGCGGCAC 313
     |  ||  |||||  |||||  ||  ||  ||  |||||  ||  ||  ||   |  ||  ||
 251 CAGAAAACATAAACAATATTACGTCGCCCTCACCTGGGTCAAGACGTCAT 300

 314 AAGCGGTTCGCCGGCATCGCCATCGGCATCGCCGCCCTGGGCGTGGCCAC 363
     ||  |||||  ||  |||||  |||||  |||||  ||  |||||  ||  ||  ||  ||
 301 AAACGGTTTGCTGGCATTGCCATTGGCATTGCgGCcCTCGGTGTTGCGAC 350

 364 CGCCGCCCAGGTGACCGCCGCCGTGTCCCTGGTGCAGGCCCAGACCAACG 413
     |||  ||  ||  |||||  |||||  ||  ||   |  ||  ||  ||  |||||  ||  |
 351 CGCAGCACAAGTGACTGCCGCTGTCTCATTAGTTCAAGCACAGACAAATG 400

 414 CCCGCGCCATCGCCGCCATGAAGAACTCCATCCAGGCCACCAACCGCGCC 463
     |  ||  ||  ||  ||  ||  |||||  ||  ||  ||  |||||  ||  ||  ||  ||
 401 CACGTGCAATAGCAGCGATGAAAAATTCAATACAGGCAACTAATCGGGCA 450

 464 GTGTTCGAGGTGAAGGAGGGCACCCAGCAGCTGGCCATCGCCGTGCAGGC 513
     ||  |||||  ||||||||  ||||||||  |||  |  ||  ||  ||  ||  ||  ||
 451 GTCTTCGAAGTGAAGGAAGGCACCCAACAGTTAGCTATAGCGGTACAAGC 500

 514 CATCCAGGACCACATCAACACCATCATGAACACCCAGCTGAACAACATGT 563
     ||||||  |||||  |||||  ||  ||  ||||||||||  |||||||  ||||
 501 cATcCAAGACCATATCAATACTATTATGAACACCCAATTGAACAATATGT 550
```

```
564 CCTGCCAGATCCTGGACAACCAGCTGGCCACCTCCCTGGGCCTGTACCTG 613
    | || |||||||| || |||||||| || |||||||| || | |||||
551 CTTGTCAGATCCTTGATAACCAGCTTGCAACCTCCCTAGGATTATACCTA 600

614 ACCGAGCTGACCACCGTGTTCCAGCCCCAGCTGATCAACCCCGCCCTGtc 663
    || ||  | || || ||||| ||||| ||  | || || || ||  ||||
601 ACAGAATTAACAACAGTGTTTCAGCCACAATTAATTAATCCAGCATTGTC 650

664 cCCCATCAGTATCCAGGCCCTGCGGTCCCTGCTGGGCAGCATGACCCCCG 713
     || || ||||| || ||| || ||||  |||| || || ||||| || |
651 ACCGATTAGTATACAAGCCTTGAGGTCTTTGCTTGGAAGTATGACACCTG 700

714 CCGTGGTGCAGGCCACCCTGAGCACCTCCATCAGCGCCGCCGAGATCCTG 763
    | ||||| || || ||  |    || || ||    || || || || ||
701 CAGTGGTTCAAGCAACATTATCTACTTCAATTTCTGCTGCTGAAATACTA 750

764 AGCGCCGGCCTGATGGAGGGCCAGATCGTGTCCGTGCTGCTGGACGAGAT 813
    || ||||| || |||||||| ||||| || || || ||||| || |||||
751 AGTGCCGGTCTAATGGAGGGTCAGATAGTTTCTGTTCTGCTAGATGAGAT 800

814 GCAGATGATCGTGAAGATCAACGTGCCCACCATCGTGACCCAGTCCAACG 863
    ||||||||| || ||||| ||||| || ||||| || || || || || |
801 GCAGATGATAGTTAAGATAAACGTTCCAACCATTGTCACACAATCAAATG 850

864 CCCTGGTGATCGACTTCTACAGCATCAGCAGCTTCATCAACAACCAGGAG 913
    |  ||||||| |||||||||   ||    || || || || || || ||
851 CATTGGTGATTGACTTCTACTCAATTTCGAGTTTTATTAATAATCAAGAA 900

914 TCCATCATCCAGCTGCCCGACCGCATCCTGGAGATCGGCAACGAGCAGTG 963
    ||||| || ||  |||| ||| | ||| |||||||||| || || || ||
901 TCCATAATTCAATTGCCAGACAGGATCTTGGAGATCGGAAATGAACAATG 950

964 GCGCTACCCCGCCAAGAACTGCAAGCTGACCCGCCACCACATCTTCTGCC 1013
    |||||| || || ||||| || ||| ||||  | |||||||| |||||||
951 GCGCTATCCAGCTAAGAATTGTAAGTTGACAAGACACCACATATTCTGCC 1000

1014 AGTACAACGAGGCCGAGCGGCTGAGCCTGGAGACCAAGCTGTGCCTGGCC 1063
     | ||||| ||||| ||| |||||||||| || || || || ||||| ||
1001 AATACAATGAGGCAGAGAGGCTGAGCCTAGAAACAAAACTATGCCTTGCA 1050

1064 GGCAACATCAGCGCCTGCGTGTTCTCCAGCATCGCCGGCAGCTACATGCG 1113
     ||||| || || ||||| |||||||     || || || || || ||| |
1051 GGCAATATTAGTGCCTGTGTGTTCTCATCTATAGCAGGGAGTTATATGAG 1100

1114 CCGCTTCGTGGCCCTGGACGGCACCATCGTGGCCAACTGCCGCAGCCTGA 1163
      || || || || ||||| || || || || || ||||| || || || |
1101 GCGATTTGTAGCACTGGATGGAACAATTGTTGCAAACTGTCGAAGTCTAA 1150

1164 CCTGCCTGTGCAAGAGCCCCTCCTACCCCATCTACCAGCCCGACCACCAC 1213
     | || || |||||||| || || || || || ||||| || ||||| ||
1151 CGTGTCTATGCAAGAGTCCATCTTATCCTATATACCAACCTGACCATCAT 1200
```

```
1864 CATCCGGGGCCAGTTCAGCAACATGAGCCTGTCCCTGCTGGACCTGTACC 1913
1851 AATCAGAGGTCAATTCTCAAACATGTCGCTGTCCCTGTTAGACTTGTATT 1900

1914 TGGGCCGGGGCTACAACGTGAGCAGCATCGTGACCATGACCAGCCAGGGC 1963
1901 TAGGTCGAGGTTACAATGTGTCATCTATAGTCACTATGACATCCCAGGGA 1950

1964 ATGTACGGCGGCACCTACCTGGTGGAGAAGCCCAACCTGAGCAGCAAGCG 2013
1951 ATGTATGGGGGAACTTACCTAGTGGAAAAGCCTAATCTGAGCAGCAAAAG 2000

2014 GAGCGAGCTGAGCCAGCTGAGCATGTACCGCGTGTTCGAGGTGGGCGTGA 2063
2001 GTCAGAGTTGTCACAACTGAGCATGTACCGAGTGTTTGAAGTAGGTGTTA 2050

2064 TCCGGAACCCCGGCCTGGGCGCCCCCGTGTTCCACATGACCAACTACCTG 2113
2051 TCAGAAATCCGGGTTTGGGGGCTCCGGTGTTCCATATGACAAACTATCTT 2100

2114 GAGCAGCCCGTGAGCAACGACCTGAGCAACTGCATGGTGGCCCTGGGCGA 2163
2101 GAGCAACCAGTCAGTAATGATCTCAGCAACTGTATGGTGGCTTTGGGGGA 2150

2164 GCTGAAGCTGGCCGCCCTGTGCCACGGCGAGGACAGCATCACCATCCCCT 2213
2151 GCTCAAACTCGCAGCCCTTTGTCACGGGGAAGATTCTATCACAATTCCCT 2200

2214 ACCAGGGCAGCGGCAAGGGCGTGAGCTTCCAGCTGGTGAAGCTGGGCGTG 2263
2201 ATCAGGGATCAGGGAAAGGTGTCAGCTTCCAGCTCGTCAAGCTAGGTGTC 2250

2264 TGGAAGAGCCCCACCGACATGCAGAGCTGGGTGCCCCTGAGCACCGACGA 2313
2251 TGGAAATCCCCAACCGACATGCAATCCTGGGTCCCCTTATCAACGGATGA 2300

2314 CCCCGTGATCGACCGCCTGTACCTGAGCAGCCACCGCGGCGTGATCGCCG 2363
2301 TCCAGTGATAGACAGGCTTTACCTCTCATCTCACAGAGGTGTTATCGCTG 2350

2364 ACAACCAGGCCAAGTGGGCCGTGCCCACCACCCGCACCGACGACAAGCTG 2413
2351 ACAAcCAAGCAAAATGGGCTGTCCCGACAACACGAACAGATGACAAGTTG 2400

2414 CGCATGGAGACCTGCTTCCAGCAGGCCTGCAAGGGCAAGATCCAGGCCCT 2463
2401 CGAATGGAGACATGCTTCCAACAGGCGTGTAAGGGTAAAATCCAAGCACT 2450

2464 GTGCGAGAACCCCGAaTGGGCCCCCCTGAAGGACAACCGCATCCCCAGCT 2513
2451 CTGCGAGAATCCCGAGTGGGCACCATTGAAGGATAACAGGATTCCTTCAT 2500
```

```
2514 ACGGCGTGCTGAGCGTGGACCTGAGCCTGACCGTGGAGCTGAAGATCAAG 2563
     |||| ||  ||   || || ||||| ||||| || ||||| || ||||||
2501 ACGGGGTCTTGTCTGTTGATCTGAGTCTGACAGTTGAGCTTAAAATCAAA 2550

2564 ATCGCGAGCGGCTTCGGCCCCCTGATCACCCACGGCAGCGGCATGGACCT 2613
     || ||    || ||||| ||  ||||||| |||||    || ||||||||
2551 ATTGCTTCGGGATTCGGGCCATTGATCACACACGGTTCAGGGATGGACCT 2600

2614 GTACAAGAGCAACCACAACAACGTGTACTGGCTGACCATCCCCCCCATGA 2663
      |||||   |||||||||||| ||||| |||||||| ||||| || ||||
2601 ATACAAATCCAACCACAACAATGTGTATTGGCTGACTATCCCGCCAATGA 2650

2664 AGAACCTGGCCCTGGGCGTGATCAACACCCTGGAGTGGATtCCCCGCTTC 2713
     ||||||| ||| | || || |||||||| |||||||||| ||  | |||
2651 AGAACCTAGCCTTAGGTGTAATCAACACATTGGAGTGGATACCGAGATTC 2700

2714 AAGGTGAGCCCCTACCTGTTCACCGTGCCCATCAAGGAGGCCGGCGAGGA 2763
     ||||| || |||||||| ||||| || || || ||||| || ||||| ||
2701 AAGGTTAGTCCCTACCTCTTCAcTGTCCCAATTAAGGAAGCAGGCGAAGA 2750

2764 CTGCCACGCCCCGACCTACCTGCCCGCCGAGGTGGACGGCGACGTGAAGC 2813
     |||||| ||||| || ||||| || || |||||||| || || || || |
2751 CTGCCATGCCCCAACATACCTACCTGCGGAGGTGGATGGTGATGTCAAAC 2800

2814 TGAGCAGCAACCTGGTGATCCTGCCCGGCCAGGACCTGCAGTACGTGCTG 2863
     | ||   ||| |||||||| || || || || || || || || ||  ||
2801 TCAGTTCCAATCTGGTGATTCTACCTGGTCAAGATCTCCAATATGTTTTG 2850

2864 GCCACCTACGACACCAGCCGCGTGGAGCACGCCGTGGTGTACTACGTGTA 2913
     || |||||||| ||   | | || || || || ||||| || ||||| ||
2851 GCAACCTACGATACTTCCAGGGTTGAACATGCTGTGGTTTATTACGTTTA 2900

2914 CAGCCCCGGCCGCAGCTTCTTCTACTTCTACCCCTTCCGCCTGCCCATCA 2963
     |||||| ||||||   || || ||||| || || ||  |  |||| || |
2901 CAGCCCAgGCCGCTCATTTTtTTACTTTTATCCTTTTAGGTTGCCTATAA 2950

2964 AGGGCGTGCCCATCGAGCTGCAGGTGGAGTGCTTCACCTGGGACCAGAAG 3013
     |||| || |||||||| | || ||||| |||||||| |||||||| ||
2951 AGGGGGTCCCCATCGAATTACAAGTGGAATGCTTCACATGGGACCAAAAA 3000

3014 CTGTGGTGCCGCCACTTCTGCGTGCTGGCCGACAGCGAGAGCGGCGGCCA 3063
     || |||||||| |||||||| ||||| || |||   ||    || || ||
3001 CTCTGGTGCCGTCACTTCTGTGTGCTTGCGGACTCAGAATCTGGTGGACA 3050

3064 CATCACCCACAGCGGCATGGTGGGCATGGGCGTGAGCTGCACCGTGACCC 3113
      ||||| |||   || |||||||||||||| || |||||||| || ||||
3051 TATCACTCACTCTGGGATGGtGGGCATGGGAGTCAGCTGCACAGTCACCC 3100

3114 GCGAGGACGGCACCAACCGCCGCTAG 3139
     | || || || ||||| ||| | |||
3101 GGGAAGATGGAACCAATCGCAGATAG 3126
```

Fig. 42B : $F_{MU}H_{MV}$.seq check: 4381 from: 1 to: 3126
nucleic acid sequence of $F_{MU}H_{MV}$(non humanised)

ATGAAGGCTTTTCCAGTTATTTGCTTGGGCTTTGCAATCTTTTCATCCTC

TATATGTGTGAATATCAATATCTTGCAGCAAATTGGATACATCAAGCAAC

AGGTCAGGCAACTAAGCTATTACTCACAAAGTTCAAGCTCCTACGTAGTG

GTCAAGCTTTTACCGAATATCCAACCCACTGATAACAGCTGTGAATTTAA

GAGTGTAACTCAATACAATAAGACCTTGAGTAATTTGCTTCTTCCAATTG

CAGAAAACATAAACAATATTACGTCGCCCTCACCTGGGTCAAGACGTCAT

AAACGGTTTGCTGGCATTGCCATTGGCATTGCgGCcCTCGGTGTTGCGAC

CGCAGCACAAGTGACTGCCGCTGTCTCATTAGTTCAAGCACAGACAAATG

CACGTGCAATAGCAGCGATGAAAAATTCAATACAGGCAACTAATCGGGCA

GTCTTCGAAGTGAAGGAAGGCACCCAACAGTTAGCTATAGCGGTACAAGC

cATcCAAGACCATATCAATACTATTATGAACACCCAATTGAACAATATGT

CTTGTCAGATCCTTGATAACCAGCTTGCAACCTCCCTAGGATTATACCTA

ACAGAATTAACAACAGTGTTTCAGCCACAATTAATTAATCCAGCATTGTC

ACCGATTAGTATACAAGCCTTGAGGTCTTTGCTTGGAAGTATGACACCTG

CAGTGGTTCAAGCAACATTATCTACTTCAATTTCTGCTGCTGAAATACTA

AGTGCCGGTCTAATGGAGGGTCAGATAGTTTCTGTTCTGCTAGATGAGAT

GCAGATGATAGTTAAGATAAACGTTCCAACCATTGTCACACAATCAAATG

CATTGGTGATTGACTTCTACTCAATTTCGAGTTTTATTAATAATCAAGAA

TCCATAATTCAATTGCCAGACAGGATCTTGGAGATCGGAAATGAACAATG

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AATACAATGAGGCAGAGAGGCTGAGCCTAGAAACAAAACTATGCCTTGCA

GGCAATATTAGTGCCTGTGTGTTCTCATCTATAGCAGGGAGTTATATGAG

GCGATTTGTAGCACTGGATGGAACAATTGTTGCAAACTGTCGAAGTCTAA

CGTGTCTATGCAAGAGTCCATCTTATCCTATATACCAACCTGACCATCAT

GCAGTCACGACCATTGATCTAACGTCATGTCAAACATTGTCCCTGGACGG

ACTGGATTTCAGCATTGTCTCGCTAAGCAACATCACTTACGCTGAGAATC

TTACTATTTCATTGTCTCAGACAATCAATACTCAACCCATTGATATATCA

ACTGAGCTGAGTAAGGTTAATGCATCCCTCCAAAATGCCGTTAAATACAT

AAAAGAGAGTAACCATCAACTCCAATCCGTTAGTGTAAGTTCTAAAAGAC

TTCATCGGGCAGCCATCTACACCGCAGAGATCCATAAAAGCCTCAGCACC

AATCTAGATGTAACTAACTCAATCGAGCATCAGGTCAAGGACGTGCTGAC

ACCACTCTTCAAAATCATCGGTGATGAAGTGGGCCTGAGGACACCTCAGA

GATTCACTGACCTAGTGAAATTCATCTCTGACAAGATTAAATTCCTTAAT

CCGGATAGGGAGTACGACTTCAGAGATCTCACTTGGTGTATCAACCCGCC

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AAGAGCTCATGAATGCATTGGTGAACTCAACTCTACTGGAGACCAGAACA

ACCAATCAGTTCCTAGCTGTCTCAAAGGGAAACTGCTCAGGGCCCACTAC

AATCAGAGGTCAATTCTCAAACATGTCGCTGTCCCTGTTAGACTTGTATT

TAGGTCGAGGTTACAATGTGTCATCTATAGTCACTATGACATCCCAGGGA

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GTCAGAGTTGTCACAACTGAGCATGTACCGAGTGTTTGAAGTAGGTGTTA

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GAGCAACCAGTCAGTAATGATCTCAGCAACTGTATGGTGGCTTTGGGGGA

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TCCAGTGATAGACAGGCTTTACCTCTCATCTCACAGAGGTGTTATCGCTG

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CGAATGGAGACATGCTTCCAACAGGCGTGTAAGGGTAAAATCCAAGCACT

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ATTGCTTCGGGATTCGGGCCATTGATCACACACGGTTCAGGGATGGACCT

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AAGGTTAGTCCCTACCTCTTCAcTGTCCCAATTAAGGAAGCAGGCGAAGA

CTGCCATGCCCCAACATACCTACCTGCGGAGGTGGATGGTGATGTCAAAC

TCAGTTCCAATCTGGTGATTCTACCTGGTCAAGATCTCCAATATGTTTTG

GCAACCTACGATACTTCCAGGGTTGAACATGCTGTGGTTTATTACGTTTA

CAGCCCAgGCCGCTCATTTTtTTACTTTTATCCTTTTAGGTTGCCTATAA

AGGGGGTCCCCATCGAATTACAAGTGGAATGCTTCACATGGGACCAAAAA

CTCTGGTGCCGTCACTTCTGTGTGCTTGCGGACTCAGAATCTGGTGGACA

TATCACTCACTCTGGGATGGtGGGCATGGGAGTCAGCTGCACAGTCACCC

GGGAAGATGGAACCAATCGCAGATAG

[SEQ ID NO: 97]

Fig 42C: F_{MUV} hum$H_M$hum.seq check: 5778 from: 14 to: 3139
Humanised nucleic acids sequence of $F_{MuV}H_{Mv}$

ATGAAGGCGTTCCCCGTGATCTGCCTGGGCTTCGCCATCTTCTCCAGCAG

CATCTGCGTGAACATCAACATCCTGCAGCAGATCGGATACATCAAGCAGC

AGGTGAGGCAGCTGAGCTACTACTCCCAGAGCTCCAGCTCCTACGTGGTG

GTCAAGCTGCTGCCCAACATCCAGCCCACCGACAACAGCTGCGAGTTCAA

GAGCGTGACCCAGTACAACAAGACCCTGAGCAACCTGCTGCTGCCCATCG

CCGAGAACATCAACAACATCACCTCCCCCTCCCCCGGCTCCCGGCGGCAC

AAGCGGTTCGCCGGCATCGCCATCGGCATCGCCGCCCTGGGCGTGGCCAC

CGCCGCCCAGGTGACCGCCGCCGTGTCCCTGGTGCAGGCCCAGACCAACG

CCCGCGCCATCGCCGCCATGAAGAACTCCATCCAGGCCACCAACCGCGCC

GTGTTCGAGGTGAAGGAGGGCACCCAGCAGCTGGCCATCGCCGTGCAGGC

CATCCAGGACCACATCAACACCATCATGAACACCCAGCTGAACAACATGT

CCTGCCAGATCCTGGACAACCAGCTGGCCACCTCCCTGGGCCTGTACCTG

ACCGAGCTGACCACCGTGTTCCAGCCCCAGCTGATCAACCCCGCCCTGtc

cCCCATCAGTATCCAGGCCCTGCGGTCCCTGCTGGGCAGCATGACCCCCG

CCGTGGTGCAGGCCACCCTGAGCACCTCCATCAGCGCCGCCGAGATCCTG

AGCGCCGGCCTGATGGAGGGCCAGATCGTGTCCGTGCTGCTGGACGAGAT

GCAGATGATCGTGAAGATCAACGTGCCCACCATCGTGACCCAGTCCAACG

CCCTGGTGATCGACTTCTACAGCATCAGCAGCTTCATCAACAACCAGGAG

TCCATCATCCAGCTGCCCGACCGCATCCTGGAGATCGGCAACGAGCAGTG

GCGCTACCCCGCCAAGAACTGCAAGCTGACCCGCCACCACATCTTCTGCC

AGTACAACGAGGCCGAGCGGCTGAGCCTGGAGACCAAGCTGTGCCTGGCC

GGCAACATCAGCGCCTGCGTGTTCTCCAGCATCGCCGGCAGCTACATGCG

CCGCTTCGTGGCCCTGGACGGCACCATCGTGGCCAACTGCCGCAGCCTGA

CCTGCCTGTGCAAGAGCCCCTCCTACCCCATCTACCAGCCCGACCACCAC

GCCGTGACCACCATCGACCTGACCTCCTGCCAGACCCTGAGCCTGGACGG

CCTGGACTTCAGCATCGTGTCCCTGAGCAACATCACCTACGCCGAGAACC

TGACCATCAGCCTGAGCCAGACCATCAACACCCAGCCCATCGACATCTCC

ACCGAGCTGAGCAAGGTGAACGCCTCCCTGCAGAACGCCGTGAAGTACAT

CAAGGAGAGCAACCACCAGCTGCAGAGCGTGAGCGTGAGCAGCAAGCGCC

TGCACCGCGCCGCCATCTACACCGCCGAGATCCACAAGAGCCTGAGCACC

AACCTGGACGTGACCAACTCCATCGAGCACCAGGTGAAGGACGTGCTGAC

CCCCCTGTTCAAGATCATCGGCGACGAGGTGGGCCTGCGCACCCCCCAGC

GCTTCACCGACCTGGTGAAGTTCATCTCCGACAAGATCAAGTTCCTGAAC

CCCGACCGCGAGTACGACTTCCGCGACCTGACCTGGTGCATCAACCCCCC

CGAGCGGATCAAGCTGGACTACGACCAGTACTGCGCCGACGTGGCCGCCG

AGGAGCTGATGAACGCCCTGGTGAACAGCACCCTGCTGGAGACCCGCACC

ACCAACCAGTTCCTGGCCGTGAGCAAGGGCAACTGCAGCGGCCCCACCAC

CATCCGGGGCCAGTTCAGCAACATGAGCCTGTCCCTGCTGGACCTGTACC

TGGGCCGGGGCTACAACGTGAGCAGCATCGTGACCATGACCAGCCAGGGC

ATGTACGGCGGCACCTACCTGGTGGAGAAGCCCAACCTGAGCAGCAAGCG

GAGCGAGCTGAGCCAGCTGAGCATGTACCGCGTGTTCGAGGTGGGCGTGA

TCCGGAACCCCGGCCTGGGCGCCCCCGTGTTCCACATGACCAACTACCTG

GAGCAGCCCGTGAGCAACGACCTGAGCAACTGCATGGTGGCCCTGGGCGA

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CGCATGGAGACCTGCTTCCAGCAGGCCTGCAAGGGCAAGATCCAGGCCCT

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AGAACCTGGCCCTGGGCGTGATCAACACCCTGGAGTGGATtCCCCGCTTC

AAGGTGAGCCCCTACCTGTTCACCGTGCCCATCAAGGAGGCCGGCGAGGA

CTGCCACGCCCCGACCTACCTGCCCGCCGAGGTGGACGGCGACGTGAAGC

TGAGCAGCAACCTGGTGATCCTGCCCGGCCAGGACCTGCAGTACGTGCTG

GCCACCTACGACACCAGCCGCGTGGAGCACGCCGTGGTGTACTACGTGTA

CAGCCCCGGCCGCAGCTTCTTCTACTTCTACCCCTTCCGCCTGCCCATCA

AGGGCGTGCCCATCGAGCTGCAGGTGGAGTGCTTCACCTGGGACCAGAAG

CTGTGGTGCCGCCACTTCTGCGTGCTGGCCGACAGCGAGAGCGGCGGCCA

CATCACCCACAGCGGCATGGTGGGCATGGGCGTGAGCTGCACCGTGACCC

GCGAGGACGGCACCAACCGCCGCTAG

[SEQ ID NO: 98]

Fig. 43 Total IgG directed against the FHN protein

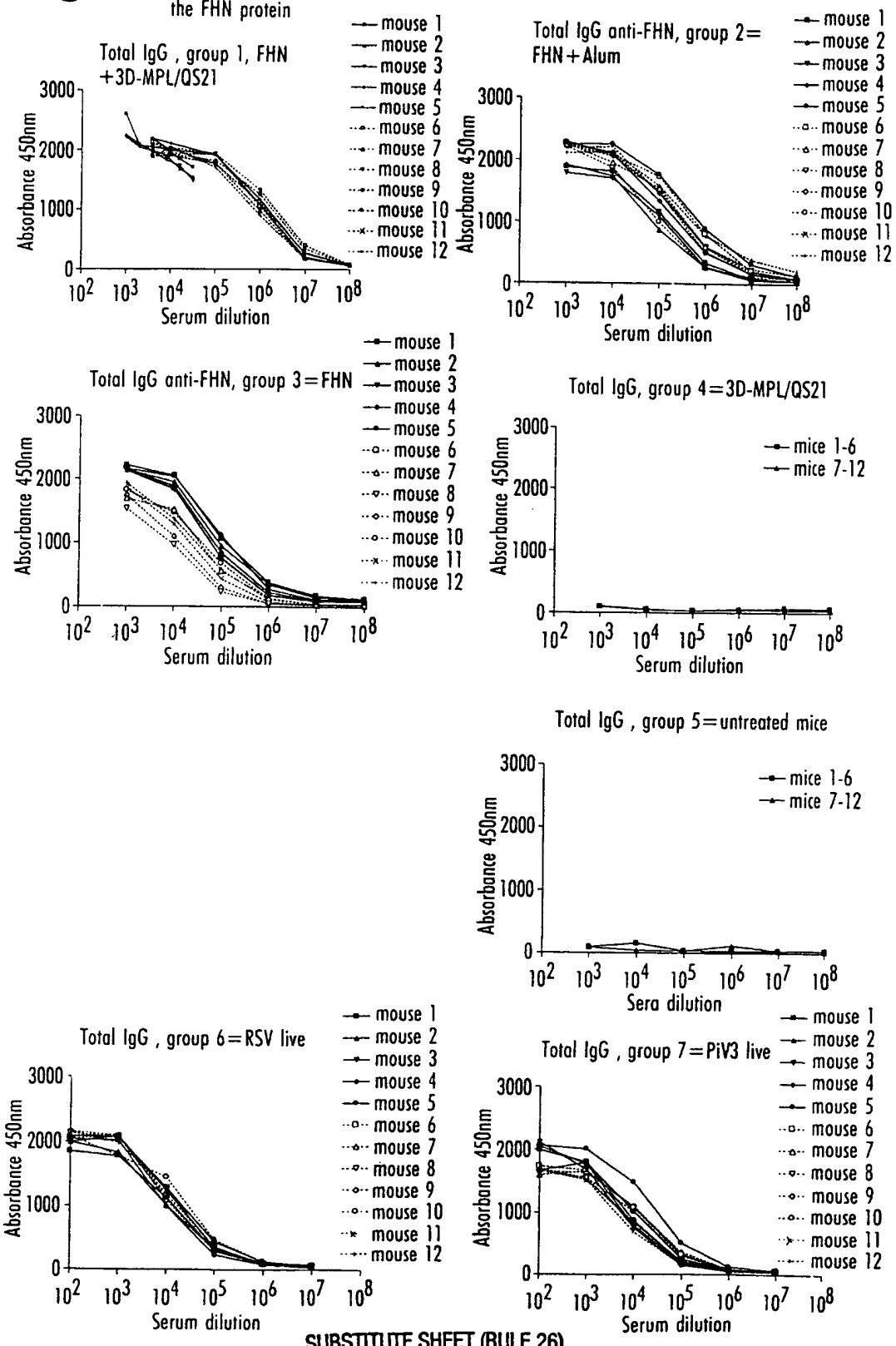

Fig. 44 IgG1, directed against the FHN protein.

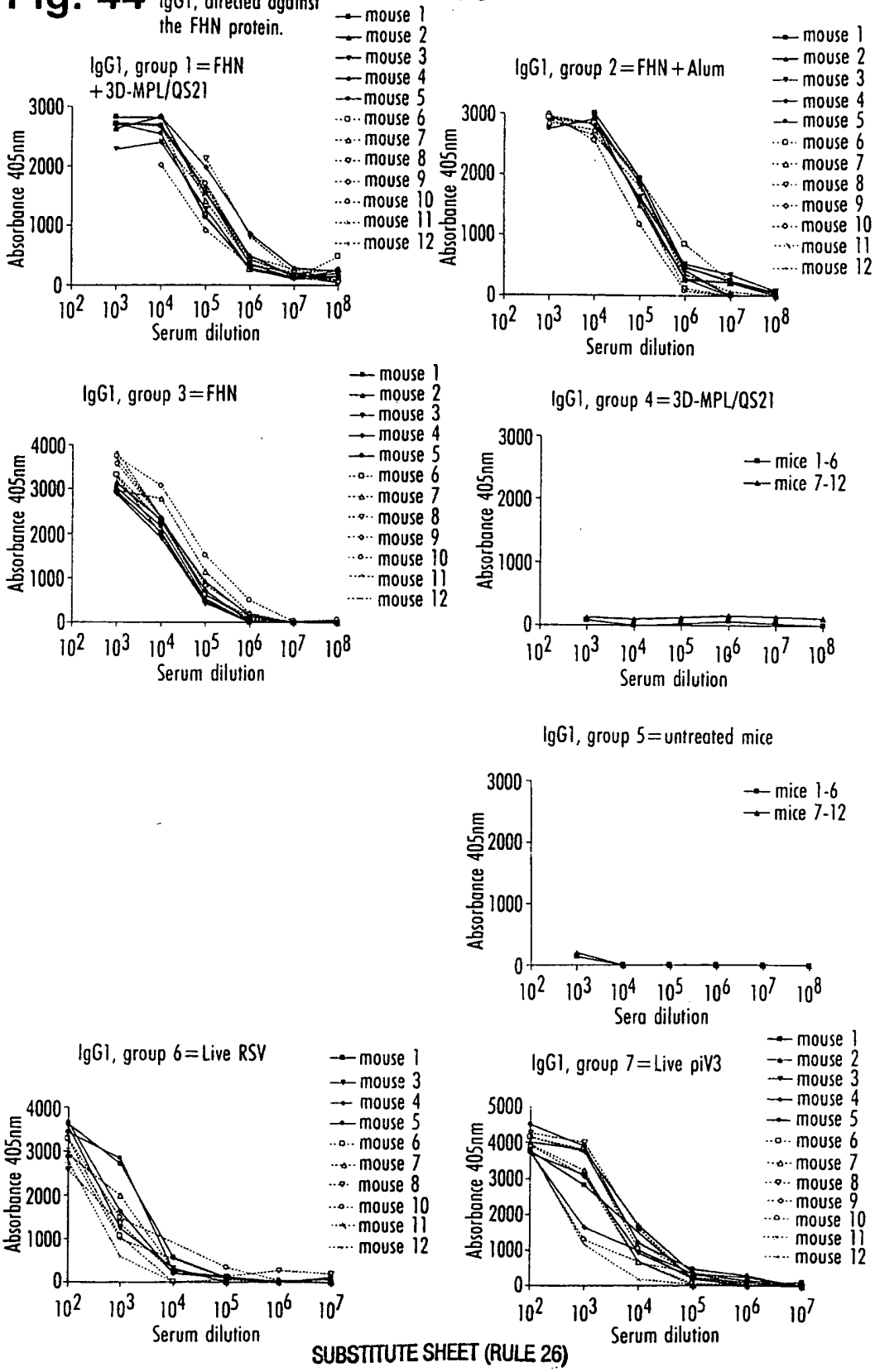

Fig. 45 IgG2a directed against the FHN protein